REPORTS

von Hippel–Lindau Gene Mutations in *N*-Nitrosodimethylamine-Induced Rat Renal Epithelial Tumors

Yih-Horng Shiao, Jerry M. Rice, Lucy M. Anderson, Bhalchandra A. Diwan, Gordon C. Hard

Background: **Mutations in the von Hippel–Lindau (VHL) gene are common in human clear cell kidney cancers. Carcinogens in cigarette smoke, especially nitrosamines, are known to induce kidney tumors of a variety of histologic types in rodents—but with no evidence of VHL mutations; however, none of these tumors resembled human clear cell carcinomas. We examined *N*-nitrosodimethylamine-induced kidney tumors of the clear or mixed clear/granular cell type in Wistar rats to assess the presence of VHL mutations.** ***Methods:*** **Sections of eight clear or mixed clear/granular cell kidney tumors that had been formalin fixed and paraffin embedded were microdissected. DNA was extracted from the microdissected tissue, and exons 1–3 of the rat VHL gene were examined by use of polymerase chain reaction and cycle sequencing techniques.** ***Results:*** **Four VHL gene mutations (three G:C to A:T and one A:T to G:C) were detected in three of the tumors in contrast to no mutations in 40 previously reported rat kidney tumors of other histologic types (three of eight tumors versus none of 40; two-sided Fisher's exact test; $P = .003$). Only tumors showing prominent swollen clear cell cytology with a signet-ring appearance had VHL mutations.** ***Conclusions:*** **To our knowledge, this is the first report of VHL mutations in kidney tumors after direct chemical exposure and provides a possible molecular pathway linking tobacco smoking to kidney cancer. [J Natl Cancer Inst 1998;90:1720–3]**

Mutations in the von Hippel–Lindau (VHL) gene are frequently detected in human renal cell carcinomas, especially of the clear cell type *(1).* Sporadic renal cell carcinomas are associated with tobacco smoking *(2),* and, of the carcinogens in cigarette smoke, the nitrosamines, especially *N*-nitrosodimethylamine (NDMA), are kidney carcinogens in rodents *(3).* Previously, we and others *(4–6)* have examined the VHL gene in chemically induced and spontaneous rat kidney tumors and cell lines; no mutations were detected in a variety of tumor histologic types. However, none of these tumors resembled histologically the clear cell carcinomas common in humans.

In this study, we examined the VHL gene in kidney tumors of clear or mixed clear/granular cell type induced in protein-deprived Wistar rats by intraperitoneal injection of NDMA. These tumors, although rare in animals, were histologically similar to the common form of human renal cell carcinomas. We therefore hypothesized that these rat kidney tumors might present VHL mutations.

Materials and Methods

Archived Tissues

The tumors examined in this study represented archival materials, fixed in formalin and embedded in paraffin, from experiments conducted more than 10 years ago on renal tumor pathogenesis. These early studies were based on a high incidence model of renal epithelial tumors using NDMA as the carcinogen, as previously described *(7).* In brief, prior to an intraperitoneal injection of NDMA, female Crl: (W)BR Wistar rats (Charles River Laboratories, Wilmington, MA) were fed a powdered diet consisting exclusively of glucose:sucrose (50:50) mixture with 20% aqueous glucose solution as drinking water for 3–5 days. This pretreatment of protein deprivation is known to reduce the liver's capacity to metabolize NDMA, resulting in increased dose delivery to the kidneys *(8,9).* With this regimen, epithelial tumors derived from the renal tubules can be induced in up to 90% of dosed rats. The tumors are mainly of the granular cell type, with only a low percentage of clear cell or mixed clear/granular cell type (about 10%). The eight clear or mixed clear/granular tumors (Table 1) selected for VHL mutation analysis were from female Wistar rats that had received either one (tumors T1, T2, T5, T7, and T8) or two (tumors T3, T4, and T6) intraperitoneal injections of NDMA (30 mg/kg body weight). The first NDMA injection was administered at 10 weeks of age after 5 days of protein deprivation and the second injection at 12 weeks of age after 4 days of protein deprivation. After each NDMA injection, the rats were returned to a conventional pelleted diet with water *ad libitum.* The eight tumors were either detected by abdominal palpation or found at necropsy during the treatment periods of 79–109 weeks. Hematoxylin–eosin-stained sections were used for morphologic examination.

DNA Extraction and Polymerase Chain Reaction (PCR)

One to three 10-μm paraffin sections were used for microdissection and neoplastic areas were pooled for DNA extraction according to methodology described previously *(4).* Intron-derived primer sets (E1a: 5′-TCCGGAGGCGTCCGGTTC-3′/5′-GCGAGTTCACAGAGCGTAAAA-3′; E1b: 5′-GGCTCTGAAGAGATAGAGG-3′/5′-TCAAAGTTGAGCCACAAAG-3′; E1c: 5′-AGCCCGCGCGTCGTGCT-3′/5′-GGCC-TAGCGCGCGTCTCG-3′; E2a: 5′-CATCCTCT-GTTTGCATTGAC-3′/5′-AATAGGCTGTCCAT-CAACAT-3′; E2b: 5′-GGAACTGTTTGTGC-CATC-3′/5′-CCCAAGGTCTTATTTCATAA-3′; E3a: 5′-GAGCCTGCCTCAGAGGACTT-3′/5′-CTGACGATGTCCAGCCTCCT-3′; E3b: 5′-GCCTGGTCAAGCCTGAGAAC-3′/5′-TCGAGGTGCTCTTGGGTCAG-3′; E3c: 5′-CCCAAATGTGCGGAAAGACAT-3′/5′-CCATCAAGGCCGAAATTCAG-3′) were selected from previously reported intron sequences *(4)* to amplify coding regions and exon-intron boundaries of exons 1–3 of the rat VHL gene. The thermal cycling profile for PCR was modified from a previous protocol *(4).* In brief, PCR was performed as follows: 40 cycles of denaturation (94 °C for 30 seconds), annealing (55 °C, 50 °C, 65 °C, 50 °C, 50 °C, 60 °C, 65 °C, and 55 °C for 30 seconds for primers E1a, E1b, E1c, E2a, E2b, E3a, E3b, and E3c, respectively), and extension (72 °C for 1 minute) followed by a final 5-minute extension at 72 °C. For primers E1c, 5% dimethylsulfoxide was also included in PCR reactions.

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See ''Notes'' following ''References.''

Table 1. Mutations of the von Hippel–Lindau gene in *N*-nitrosodimethylamine-induced rat renal epithelial tumors*

Tumor	Gene	Codon	Base change	Amino acid	Cell type	Growth pattern
T1	—	—	—	—	Clear cells	Solid
T2	—	—	—	—	Mixed, clear cells predominate	Solid
T3	Intron 2		GTTGAT to GTTAAT		Mixed, clear cells predominate	Solid/papillary
	Exon 3	165	CAG to CAA	Gln to Gln		
T4	—	—	—	—	Mixed, granular cells predominate	Solid/papillary
T5	—	—	—	—	Mixed, clear cells predominate	Solid
T6	—	—	—	—	Mixed	Solid
T7	Exon 1	20	ATA to GTA	Ile to Val	Mixed	Solid
T8	Exon 3	142	AGG to AAG	Arg to Lys	Mixed, clear cells predominate	Solid

*Gln = glutamine; Ile = isoleucine; Val = valine; Arg = arginine; and Lys = lysine.

DNA Sequencing

Twenty microliters of PCR products was gel purified in a 6% or 10% polyacrylamide gel (Novex, San Diego, CA). Excised gel pieces were incubated with 50 m*M* of ammonium acetate for greater than 10 hours at 50 °C. Gel pieces were then removed by a 0.22-μm spin column (Millipore, Bedford, MA). DNA was precipitated by two volumes of absolute ethanol. A one-third volume of purified product was used for DNA sequencing. The same primer sets used for PCR were also used for DNA sequencing, which was performed by the dideoxynucleotide method with a Sequenase Cycle Sequencing Kit (#US79750; Amersham Life Science, Inc., Arlington Heights, IL) and ^{33}P-labeled dideoxyribonucleoside triphosphates. The sequencing reaction products were resolved in a 7.7 *M* urea/6% polyacrylamide gel. After electrophoresis, the gel was dried in a gel dryer (model 583; Bio-Rad Laboratories, Richmond, CA) and exposed to Kodak XAR-5 x-ray film (Eastman Kodak Co., New Haven, CT) for 1–3 days at room temperature. Mutations were confirmed by sequencing the same or complementary DNA strand from a second independent PCR product.

Statistical Analysis

The two-sided Fisher's exact test was used to compare the frequency of VHL mutations, if detected, among tumors that differed in histologic features.

Results

The rat clear or mixed clear/granular cell kidney tumors showed primarily a solid growth pattern, with regions of papillary growth in two tumors (T3 and T4) from the same animal (Table 1, Fig. 1). Clear cells were cytologically pleomorphic and intermingled with granular cells. Neoplastic areas were characterized by abundant blood vessels and hemorrhage. Cysts were readily detected in tumors T3, T4, T7, and T8. This histology was not observed in other tumors. Infiltration of inflammatory cells was not common in most tumors except for the T1 tumor. Sporadically distributed inflammatory and stromal cells were very limited and usually less than 5% of microdissected areas.

Four mutations were detected in three tumors (T3, T7, and T8) (Table 1). No base changes were observed in adjacent non-neoplastic tissues (data not shown). Of the mutations detected, two were missense, one was silent, and one was intronic. The intron mutation was located at the fourth position from the 5′ end of the splicing donor site. G:C to A:T transitions predominated (three of four mutations). Tumor T3 had two mutations but tumor T4 from the same animal did not have any detectable mutations.

When tumor histology was considered, a striking concordance was observed between tumor phenotype and VHL genotype. Statistical analysis showed that VHL mutations were significantly associated with rat kidney tumors of clear or mixed clear/granular cell phenotype in

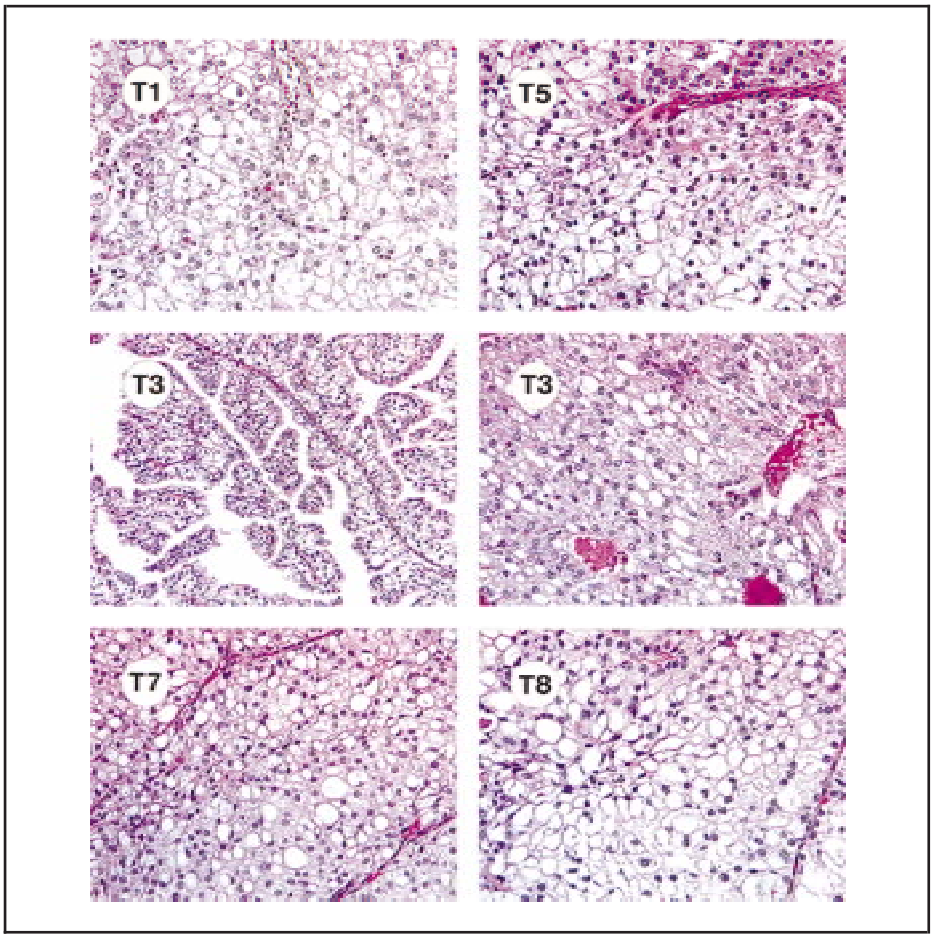

Fig. 1. Histology of *N*-nitrosodimethylamine-induced renal cell tumors (hematoxylin–eosin, original magnification ×300). Tumors T1 and T5 contain typical clear cells characterized by a polygonal contour with clear cytoplasm and eccentric nuclei. Clear cells in tumors T3 (right-center), T7, and T8 appear to be swollen and often have a signet-ring cytology. In a selected area, tumor T3 also shows a papillary growth pattern (left-center, ×150 magnification).

comparison to other phenotypes previously reported *(4,5)* (three of eight tumors versus none of 40; $P = .003$). In a subset of tumors, the three with VHL mutations (T3, T7, and T8) shared a unique histology characterized by the presence of apparently swollen clear cells with nuclei pushed against the cell membrane, often giving a signet-ring cell phenotype (Fig. 1). No VHL mutations were detected in the remaining five tumors, in which the swollen clear cell phenotype was absent or negligible. The association between VHL mutation and the swollen clear cell phenotype was also statistically significant (three of three tumors versus none of five; $P = .02$).

DNA sequencing data showed only a mutant allele in tumors T7 and T8 (i.e., wild-type alleles were absent), although both clear and granular cells were present (Fig. 2). Tumor T3 presented both mutant and wild-type bands in DNA sequencing analysis. This tumor had a papillary growth pattern in some areas, in contrast to a uniform solid growth pattern in tumors T7 and T8.

Sequencing analysis of the Wistar rat VHL gene also detected a possible genetic polymorphism consisting of deletion of the seventh base following the stop codon (TGA) in comparison to a VHL sequence (accession number U14746) from GenBank. The 3′ sequence from TGA is 5′-GATTACTGGT-3′, one base (T) less than the reported 5′-GATTACTTGGT-3′ (data not shown).

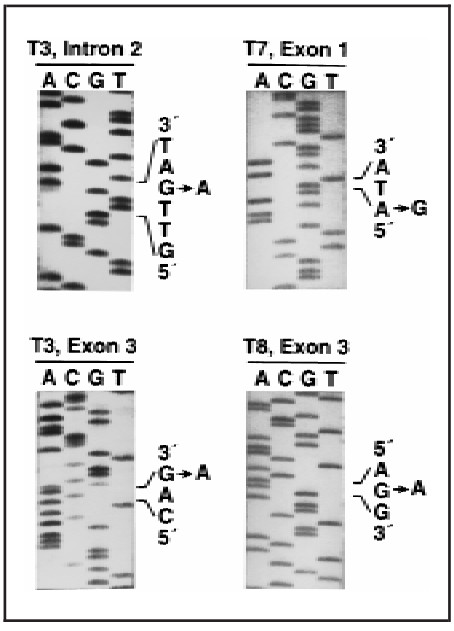

Fig. 2. DNA sequencing analyses showing von Hippel-Lindau gene mutations in tumors T3, T7, and T8. Wild-type sequences are shown directly adjacent to the sequencing ladders and individual nucleotide changes (mutations) are indicated by use of arrows.

Discussion

Predominant G:C to A:T mutations in these tumors are consistent with a previous report *(10)* of a persistence of O^6-methylguanine, which often results in G to A base change *(11)* in NDMA-treated rat kidneys. The other mutation, A:T to G:C transition, has also been reported in other *in vivo* systems after treatment with NDMA *(12)*. In human sporadic renal cell carcinomas, 30%–70% of base substitution mutations in VHL are G:C to A:T or A:T to G:C transitions *(13–16)*. Our findings are consistent with a possible role of NDMA, a carcinogen detected in tobacco smoke *(17)*, in induction of VHL mutations and causation of renal tumors. In contrast, VHL frameshift mutations, which occur frequently in human sporadic renal cell carcinomas (>50% of VHL mutations) *(13–16)*, were not observed in these NDMA-induced rat renal epithelial tumors. The rat model may be useful for discerning the types of chemical insults that lead to these changes.

Of the VHL mutations found in our rat kidney tumors, codon 142 is conserved in the human gene, whereas codons 20 and 165 are different. These codons do not correspond exactly to those mutations reported in the human VHL gene thus far *(13–16)* but are in immediate proximity. However, VHL mutations in both human and rat kidney tumors share a similarity in that mutations tend to distribute throughout exons 1–3. Additional functional study of these mutations may clarify the role of VHL in kidney tumorigenesis.

Previously, we and others *(4–6)* examined many rat kidney tumors covering a wide spectrum of histology as well as cell lines. These tumors included 23 renal epithelial tumors of granular cell type, 10 nephroblastomas, and seven renal mesenchymal tumors. DNA screening analyses for the three exons of the VHL gene did not reveal any mutations in these tumors. The finding of VHL mutations only in rat kidney tumors with clear cell phenotype in the current study is consistent with reports in humans *(1)*. Furthermore, the concordance of VHL mutations with the swollen clear cell phenotype is intriguing. It is possible that this cytology reflects a phenotypic impact of the VHL mutations, leading to the swollen clear cells with sporadic signet-ring cytology; the cellular changes underlying this phenotype remain to be discovered.

The swollen clear cell phenotype has been also described as vacuolation in previous reports of NDMA-induced rat kidney tumors *(18,19)*. The cytoplasmic composition of the swollen clear cells is unknown. Their occurrence appears not to associate with necrosis-related degeneration. Accumulation of lipid is suggested because a similar signet-ring morphology has been described in lipoma of rat kidney *(3)*. However, there was no evidence that the swollen clear cells originated from adipocytes. In previous studies, renal epithelial tumors induced by NDMA have been demonstrated histologically and ultrastructurally to be derived from renal proximal tubules *(20–22)* as are human renal cell carcinomas of mixed clear/granular cell cytology *(23–26)*. This observation suggests that the swollen cells are a subset of clear cells with morphologically distinctive cytoplasmic contents. In humans, the majority of renal carcinoma cells contain glycogen, lipid, and/or vacuoles of unknown type in variable proportions *(23)*. Accordingly, human and NDMA-induced rat renal epithelial tumors appear to have a similar pathogenesis.

The finding of only a mutant allele in tumors T7 and T8 is striking. This finding suggests that the cell population of these tumors was uniform with respect to VHL mutation and probably had undergone loss of heterozygosity (LOH). For tumor T3, detection of both mutant and wild-type alleles may relate to the coexistence of swollen clear cells and lesions with papillary growth pattern in this tumor. It is known that VHL is not mutated in the human counterpart of papillary kidney cancer *(13,16)*. Alternatively, the mechanism of tumorigenesis in tumor T3 in the presence of both mutant and wild-type alleles may involve inactivation of VHL expression from the wild-type allele by DNA methylation. As a result, the mutated VHL protein may escape from dominant-negative suppression by the wild-type VHL protein.

It has been suggested that coexistence of the clear and granular cells may be a

result of interconversion between the two cell types by shifts in intermediary metabolism *(27)*. The current observation of uniform VHL mutations in NDMA-induced mixed clear/granular cell tumors (T7 and T8) supports the rationale that mixed cytology represents different stages of tumor morphogenesis.

More than 90% of clear cell carcinomas in humans showed LOH of the region of chromosome 3p, in which VHL is located, but only 30%–60% had VHL mutations *(1)*. This finding indicates that loss of one VHL allele or other gene(s) at the same chromosomal locus may also contribute to renal carcinogenesis. LOH analysis was not performed in our study because of lack of polymorphic markers in the vicinity of the VHL gene in outbred Wistar rats. However, we did find a possible polymorphic site within the noncoding region of exon 3. The lack of one thymidine (T) in Wistar rats is different from the reported VHL sequence (GenBank accession number U14746), which is obtained from the BRL-8 complementary DNA clone of Buffalo rat origin but identical to the sequence from Long Evans rats *(5)*. In a previous sequencing analysis, we also observed a base difference at the 10th position upstream from the 3′ end of intron 1 between Fischer 344 and Long Evans rats *(4)*. If these polymorphisms are confirmed, they may be useful for determination of LOH in hybrid animals.

In summary, we examined the VHL gene in NDMA-induced renal epithelial tumors in a rat model and have found VHL mutated in a tumor type of clear or mixed clear/granular cells, histologically similar to those occurring in humans. Furthermore, the association of VHL mutation with the unique swollen clear cell phenotype provides a novel opportunity for study of the cellular effects of VHL during renal tumorigenesis.

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NOTES

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Well-Done Meat Intake and the Risk of Breast Cancer

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Background: **Heterocyclic amines, mutagens formed in meats cooked at high temperatures, have been demonstrated as mammary carcinogens in animals. We conducted a nested, case–control study among 41 836 cohort members of the Iowa Women's Health Study to evaluate the potential role of heterocyclic amines and intake of well-done meat in the risk for human breast cancer.** ***Methods:*** **A questionnaire was mailed to individuals in the cohort who had breast cancer diagnosed during the period from 1992 through 1994 and a random sample of cancer-free cohort members to obtain information on usual intake of meats and on meat preparation practices. Color photographs showing various doneness levels of hamburger, beefsteak, and bacon were included. Multivariate analysis was performed on data from 273 case subjects and 657 control subjects who completed the survey.** ***Results:*** **A dose–response relationship was found between doneness levels of meat consumed and breast cancer risk. The adjusted odds ratios (ORs) for very well-done meat versus rare or medium-done meat were 1.54 (95% confidence interval [CI] = 0.96–2.47) for hamburger, 2.21 (95% CI = 1.30–3.77) for beef steak, and 1.64 (95% CI = 0.92–2.93) for bacon. Women who consumed these three meats consistently very well done had a 4.62 times higher risk (95% CI = 1.36–15.70) than that of women who consumed the meats rare or medium done. Risk of breast cancer was also elevated with increasing intake of well-done to very well-done meat.** ***Conclusions:*** **Consumption of well-done meats and, thus, exposures to heterocyclic amines (or other compounds) formed during high-temperature cooking may play an important role in the risk of breast cancer. [J Natl Cancer Inst 1998;90:1724–9]**

High consumption of meat has been reported to be associated with an increased risk of breast cancer in some epidemiologic studies *(1)*, including several large cohort studies *(2–4)*. Dietary fat has long been suspected to be responsible for the meat–breast cancer association. This hypothesis, however, has not been supported by most prospective cohort studies *(5)*. Accumulating evidence from recent animal studies and some human studies has implicated heterocyclic amines in the pathogenesis of breast cancer *(6,7)*.

Heterocyclic amines are a group of mutagenic compounds identified in cooked foods, particularly in well-done meats and fish *(7–9)*. These compounds are formed as pyrolysis products of amino acids and proteins and are some of the most potent mutagens detected by use of the Ames test *(8–10)*. In animal studies, heterocyclic amines have been shown to increase the occurrence of various tumors, including those of the mammary glands *(10–12)*. Oral administration of 2-amino-1-methyl-6-phenylimidazo[4,5*b*]pyridine (PhIP), the most abundant carcinogenic heterocyclic amine in cooked meats, has been shown to induce mammary tumors in rats *(12,13)* in a dose–response manner. Furthermore, PhIP-induced mammary carcinomas exhibit an unusually high frequency of guanine to adenine transitions at the second base of codon 12 of the Ha-ras oncogene in F344 rats *(11)*, an unusual type of mutation observed in tumors induced by other mammary carcinogens, such as *N*-methyl-*N*-nitrosourea *(11)*, strongly suggesting that heterocyclic amines are potent mammary carcinogens.

Despite evidence from animal studies and *in vitro* experiments *(6–14)*, the potential role of heterocyclic amines as a risk factor for human breast cancer has not been appropriately investigated. Epidemiologic studies have attempted to measure the association between heterocyclic amines and risk of breast cancer, using dietary questionnaires that include consumption levels of fried or broiled meats as surrogate measures of heterocyclic amine exposure *(15–19)*. However, because the levels of heterocyclic amines in foods depend, to a large extent, on the duration and temperature of cooking, it is important to obtain information on the degree of meat doneness to estimate levels of heterocyclic amine exposure *(8,20,21)*. No epidemiologic study of diet and breast cancer has incorporated this information into the exposure assessment. To evaluate the role of well-done meat intake as a risk factor for breast cancer, we conducted a nested, case–control study of cases of breast cancer occurring during the period from 1992 through 1994 among participants in the Iowa Women's Health Study.

Subjects and Methods

Iowa Women's Health Study

Detailed descriptions of this cohort study have been published elsewhere *(22–24)*. Briefly, 41 836 Iowa women, aged 55–69 years, who completed a mailed questionnaire in January 1986, have been followed for mortality and for cancer incidence. The follow-up was accomplished through computer linkage of study participants with Iowa death certificate files, the National Death Index, and cancer diagnosis data collected by the Iowa State Health Registry, part of the Surveillance, Epidemiology, and End Results (SEER) Program[1] of the National Cancer Institute. The self-administered questionnaire used in the 1986 baseline survey included information on diet and other major risk factors for cancer. Diet was assessed by use of a semiquantitative food-frequency questionnaire almost identical to that used in the 1984 Nurses' Health Study *(25)*. Usual intake of specified portions of 127 food items was ascertained, and nutrient intake was estimated by use of the nutrient database developed for the Nurses' Health Study. Because no information was collected at the baseline survey on usual intake of meats by cooking method and usual doneness levels of meat, a case–control study with a supplementary survey was conducted during the period from 1995 through 1996 in a subset of cohort members.

Case–Control Study of Breast Cancer

Eligible case subjects for this study included all cohort members who had breast cancer diagnosed during the period from 1992 through 1994 (n =

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See ''Notes'' following ''References.''

453). A control sample of 900 women was randomly selected from 27 186 cohort members who were alive and free of cancer on January 1, 1992, and participated in the 1992 follow-up survey. Of these 900 women, 24 were excluded from the control group because they were later found either to have a breast cancer diagnosis during the period from 1992 through 1994 (n = 3) or to have been selected to participate in other Iowa Women's Health Study ancillary projects (n = 21) (eligible control subjects, n = 876). The three breast cancer patients were included in the case group.

All eligible subjects were asked to complete a self-administered food-frequency questionnaire on meat intake habits during the ''reference'' year (defined below). This questionnaire, which was developed on the basis of a questionnaire provided by Dr. Sinha for the use of in-person interviews *(26–28)*, included questions on usual intake and preparation of 15 meats. The meats are hamburgers or cheeseburgers; beefsteaks; pork chops; bacon; breakfast sausage links; breakfast sausage patties; other sausages that are cooked before eating; bratwurst; hot dogs or franks; chicken or turkey breast, thighs, legs, or wings; fish (including fried fish sandwiches, tuna); venison; game poultry; and smoked meats and fish. For each meat item, intake according to the following cooking methods was assessed: grilled or barbecued, fried, oven broiled, and prepared in other ways, as well as from ''fast food,'' if applicable. In addition, participants reported their usual preference for level of meat doneness by use of a series of color photographs that represented increasing levels of doneness of hamburger (four photographs), beefsteak (four photographs), and bacon (three photographs). Photographs were labeled only with number and represented a range of doneness levels from extremely rare to very well done. Information related to the doneness level of other 12 food items was not obtained in this study.

To minimize the potential effect of a breast cancer diagnosis on dietary intake, we obtained information for case subjects on usual dietary habits 1 year before cancer diagnosis. Because breast cancer cases were diagnosed during the period from 1992 through 1994 and dietary assessment was conducted during the period from 1995 through 1996, three reference years (1991, 1992, and 1993), corresponding to the years immediately before breast cancer diagnosis, were identified. Women in the control group of the study were also divided randomly into three corresponding groups with approximately equal sample sizes to obtain their dietary habits during these three reference years. A list of national and international events that occurred during the reference year was provided in the questionnaire to help participants recall their eating habits during that year.

Two hundred seventy-three (60.3%) of selected case subjects and 657 (75.0%) of the control subjects participated. The major reasons for nonparticipation were refusal (29.1% of case subjects and 18.7% of control subjects), inability to locate (4.9% of case subjects and 3.8% of control subjects), and death before contacting (5.7% of case subjects and 2.5% of control subjects).

Statistical Analysis

Odds ratios (ORs) were used to measure the strength of the association between exposures and cancer risk *(29)*. Unconditional logistic regression was used to control for potential confounders, assessed mostly at the baseline survey, and to derive adjusted ORs and 95% confidence intervals (CIs). Dietary intakes were adjusted for total energy intake to reduce potential variation that may have arisen from overreporting or underreporting of food intake. Trend tests for dose–response relationships across levels of each dietary variable were performed by treating ordinal-score variables (with values of 1, 2, 3, . . .) as continuous variables in logistic regression. Reported P values (two-sided) were from χ^2 tests (for categorical variables), Wilcoxon rank-sum tests (for continuous variables), and age-adjusted linear regression models using log-transferred data.

Meats were categorized by type (red versus white) and preparation method to facilitate statistical analyses. The ''all meat'' category included all 15 meats listed in the questionnaire. The ''red meat'' category included hamburgers, cheeseburgers, beefsteaks, pork chops, bacon, breakfast sausage links, breakfast sausage patties, other sausages, bratwurst, and hot dogs or franks. The ''white meat'' group included chicken or turkey breast; chicken or turkey thighs, legs, or wings; and fish. Individual meats and meat groupings were further classified by preparation method. Fried, grilled, and broiled red meats referred to the preparation methods of the aforementioned red meat category. Intake in grams was estimated for each food item included in the questionnaire. Thus, analyses using meat groups were based on the summation of the individual gram weights of the meats consumed in each category. A doneness score was also calculated to describe the eating preferences of participants on the basis of their responses to the color photographs. Doneness levels of rare or medium, well done, and very well done were given scores of 1, 2, and 3, respectively. The doneness score was defined as the sum of the doneness preferences for each of the three meat photographs. For example, a person who reported usually consuming rare or medium-done hamburger, beefsteak, and bacon received a score of 3, whereas a person reporting usual intake of all three meats as very well done received a score of 9.

RESULTS

The distributions of demographic and risk factors from the baseline survey conducted in 1986 are shown in Table 1 for all eligible subjects (left three columns) and for those who completed the supplementary questionnaire (right three columns). Among study participants, a family history of breast cancer, use of hormone replacement therapy, and waist-to-hip ratio were positively associated with risk of breast cancer (P<.05). Therefore, all analyses were adjusted for these variables. No apparent association with other variables, including dietary fat and total energy intake, was observed among study participants. Similar findings were observed from the analyses that included all eligible subjects selected for the study, suggesting that study participants represent reasonably well the eligible subject cohort in the distribution of major factors for breast cancer.

The median intake levels of major meat categories by case–control status are presented in Table 2. Total meat intake was higher among breast cancer case subjects than among control subjects. This difference was apparently due to a higher intake of red meats (17.7% case–control difference) but not white meats (2.1% difference). Because heterocyclic amines are formed primarily in fried, grilled, and broiled meats and human exposure to heterocyclic amines is primarily through intake of meats prepared by these three cooking methods, these meats were combined into one group for data analysis. There was an 18.8% (P = .06) difference in the median intake of fried, grilled, and broiled red meats between case subjects (26.5 g/day) and control subjects (22.3 g/day). Case subjects also consumed more red meats prepared in other ways than did control subjects (20.9% difference, P = .16), but intake of these meats was low, accounting for less than 15% of total red meats consumed. Compared with control subjects, case subjects had a slightly higher intake of fried, grilled, and broiled white meats (7.6%) but a lower intake of white meats prepared by other cooking methods (−5.6%). These case–control differences were not statistically significant. Intake of smoked meats and fish was low, and no apparent association with risk of breast cancer was observed. The P values from age-adjusted linear regression models using natural log-transformed data were similar to those obtained from the Wilcoxon rank-sum tests.

To evaluate the potential dose–response relationship between risk of breast cancer and intake levels of various meats, case subjects and control subjects were categorized into four groups for each meat variable listed in Table 2, according to the quartile distribution of the variable among controls; ORs for the upper three quartiles were estimated, as compared with the lowest quartile. There was a statistically significant positive association between intake of red meat and risk of breast cancer (P for trend, .02), with a 78% elevated risk observed for the highest versus the lowest intake quartile group (data not shown). High intake of fried, grilled, or broiled red meats and of other red meat preparations was associ-

Table 1. Comparison of case subjects and control subjects among postmenopausal Iowa women on selected demographic factors and risk factors for breast cancer

Characteristics at baseline survey conducted in 1986	All eligible subjects*			Subjects who completed supplementary questionnaire*		
	Case (n = 453)	Control (n = 876)	P†	Case (n = 273)	Control (n = 657)	P†
Demographic factors						
Age (y)‡	62 (58, 65)	61 (58, 64.5)	.04	61 (58, 65)	61 (58, 64)	.12
Education, %						
<High school	17.5	17.9		15.8	16.1	
High school	40.4	42.7	.62	37.4	41.7	.39
>High school	42.1	39.4		46.9	42.2	
Breast cancer risk factors						
Nondietary risk factors						
First-degree relative with breast cancer, %	16.5	9.3	<.01	17.2	10.4	<.01
Ever used hormone replacement therapy, %	43.2	37.9	.06	45.8	38.4	.04
Waist-to-hip ratio‡	0.84 (0.78, 0.91)	0.82 (0.78, 0.89)	<.01	0.83 (0.78, 0.90)	0.82 (0.77, 0.88)	.03
Age at menarche, y‡	13 (12, 14)	13 (12, 14)	.92	13 (12, 14)	13 (12, 14)	.66
Age at first live birth, y‡	22 (20, 25)	22 (20, 25)	.49	20 (20, 25)	22 (20, 25)	.94
Age at menopause, y‡	50 (45, 52)	50 (45, 52)	.69	50 (45, 52)	50 (45, 52)	.79
Nutritional factors						
Alcohol, g/day‡	0 (0, 2.1)	0 (0, 2.3)	.47	0 (0, 2.8)	0 (0, 2.6)	.86
Total energy intake, kcal/day‡	1755 (1358, 2179)	1693 (1366, 2086)	.06	1726 (1345, 2157)	1696 (1400, 2085)	.40
Total fat intake, g/day‡	67 (49, 85)	64 (48, 81)	.18	65 (49, 82)	64 (50, 81)	.60
Total fruit/vegetable intake, servings/wk‡	4.2 (3.1, 5.7)	4.0 (2.9, 5.4)	.04	4.2 (3.1, 5.8)	4.0 (3.0, 5.5)	.29

*All case and control subjects provided some dietary information in the Iowa Women's Health Study baseline survey questionnaire. Because no information on usual intake of meats by cooking method and usual doneness level was collected in the baseline survey, a supplementary survey was conducted among a subset of the Iowa Women's Health Study participants.

†*P* values (two-sided) were from the χ^2 tests (for categorical variables) or Wilcoxon rank-sum tests (for continuous variables).

‡Median (25th, 75th percentile) is presented.

Table 2. Comparison of the amount of different types of meat consumed by case subjects and control subjects

Type of meat	Median (25th, 75th percentile), g/day				
	Case subjects (n = 273)	Control subjects (n = 657)	% difference*	P†	P‡
All meats	67.0 (43.4, 95.1)	62.3 (42.2, 91.8)	7.5	.36	.39
Red meats	35.9 (20.7, 57.7)	30.5 (19.1, 49.7)	17.7	.07	.06
Fried, grilled, and broiled, combined	26.5 (14.8, 44.1)	22.3 (12.8, 39.2)	18.8	.06	.17
Prepared in other ways	5.2 (2.0, 11.1)	4.3 (1.6, 9.5)	20.9	.16	.26
Fried red meat	11.9 (5.2, 25.5)	10.6 (4.8, 22.0)	12.3	.29	.86
Grilled red meat	6.4 (2.4, 13.6)	5.4 (2.0, 11.6)	18.5	.16	.50
Broiled red meat	0.9 (0, 4.9)	0.7 (0, 4.0)	28.6	.76	.77
White meats	24.5 (14.0, 38.5)	24.0 (14.2, 41.5)	2.1	.84	.32
Chicken and turkey	16.1 (9.5, 27.1)	16.2 (8.5, 28.9)	−0.6	.81	.92
Fish	4.7 (1.9, 12.2)	7.1 (1.9, 12.2)	−33.8	.59	.68
Fried, grilled, and broiled, combined	15.5 (6.8, 26.2)	14.4 (7.3, 27.7)	0.76	.76	.41
Prepared in other ways	5.1 (1.3, 14.2)	5.4 (1.1, 13.7)	−5.6	.92	.70
Smoked meats and fish	0 (0, 1.4)	0 (0, 0.9)	0	.86	.91

*Expressed as $(\text{median}_{\text{cases}} - \text{median}_{\text{controls}})/\text{median}_{\text{controls}}$.

†*P* values (two-sided) were from the Wilcoxon rank-sum tests.

‡*P* values (two-sided) were from age-adjusted, linear regression models using log-transformed data.

ated with increased risk of breast cancer, but the trend tests were not statistically significant. No apparent association was observed for other meat groups listed in Table 2.

Hamburger, beefsteak, and bacon accounted for more than 60% of red meat intake in this study population. Information on doneness preferences for these meats was obtained, and their association with risk of breast cancer is shown in Table 3. There was a clear, positive, dose–response association between doneness levels of each of the three meats and risk of breast cancer. Women who usually consumed all three of these meats at a very well-done level had a 4.62-fold elevated risk (95% CI = 1.36–15.70) of breast cancer compared with those who usually ate these meats at a rare or medium doneness level.

Information on doneness levels and intake levels of hamburger, beefsteak, and bacon was combined to further classify the exposure status of case subjects and control subjects (Table 4). The risks of breast cancer were elevated with increasing doneness of meats in all intake groups. As previously mentioned, a high intake of red meat was related to an increased risk of breast cancer, but this positive association is less notable in comparison with that for meat doneness level.

DISCUSSION

To our knowledge, this is one of the most comprehensive epidemiologic studies to date to evaluate the hypothesis that the intake of well-done meat may be related to the risk of breast cancer. We found that a preference for consuming well-done meats was associated with an elevated risk of breast cancer in a dose–response manner. In contrast, high intake of dietary fat was not associated with risk of breast cancer in the cohort of the Iowa Women's Health Study *(22)*, and intake of red meat was only weakly associated with the risk of breast cancer in this subcohort. These findings suggest that heterocyclic amines and possibly other compounds, such as polycyclic aromatic

Table 3. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for the association of breast cancer with doneness levels of selected meats

Doneness levels	No. of case subjects*	No. of control subjects*	Age and energy adjusted OR	Multivariate adjusted† OR (95% CI)
Hamburger				
Rare or medium‡	123	345	1.00	1.00
Well-done	90	195	1.26	1.23 (0.89–1.71)
Very well-done	34	62	1.54	1.54 (0.96–2.47)
Trend test			$P = .03$	$P = .04$
Beefsteak				
Rare or medium‡	146	402	1.00	1.00
Well-done	74	161	1.27	1.22 (0.87–1.72)
Very well-done	29	35	2.26	2.21 (1.30–3.77)
Trend test			$P = .01$	$P = .01$
Bacon				
Rare or medium‡	18	62	1.00	1.00
Well-done	130	351	1.30	1.26 (0.71–2.22)
Very well-done	112	230	1.70	1.64 (0.92–2.93)
Trend test			$P = .01$	$P = .02$
Doneness score§				
3‡	6	24	1.00	1.00
4	54	200	1.13	1.14 (0.44–2.94)
5	71	155	1.89	1.90 (0.74–4.90)
6	57	103	2.3	2.28 (0.87–5.95)
7	31	80	1.59	1.56 (0.58–4.22)
8	13	18	3.04	2.89 (0.91–9.19)
9	12	11	4.53	4.62 (1.36–15.70)
Trend test			$P = .001$	$P = .001$

*Numbers do not sum to 273 (case subjects) or 657 (control subjects) because of missing data.

†Adjusted for age, total energy intake, family history of breast cancer, hormone replacement therapy, and waist-to-hip ratio.

‡Reference group.

§Doneness score was derived by adding the doneness levels (1, rare/medium; 2, well-done; and 3, very well-done) of all three meats.

Table 4. Adjusted odds ratios (ORs) for the association of breast cancer with intake and doneness levels of selected red meats*

Intake levels, by tertile	Doneness levels†		
	Rare/medium	Mostly well-done	Consistently well-done or very well-done
	No. of case subjects/control subjects		
Low	13/70	20/54	32/71
Intermediate	25/81	20/50	39/63
High	20/73	31/50	42/76
	Adjusted ORs (95% confidence interval)‡		
Low	1.00 (referent)	2.03 (0.92–4.48)	2.57 (1.23–5.35)
Intermediate	1.78 (0.84–3.77)	2.31 (1.04–5.13)	3.35 (1.63–6.90)
High	1.49 (0.68–3.27)	3.36 (1.58–7.16)	3.01 (1.47–6.17)

*Includes hamburgers, beefsteak, and bacon for which information on doneness levels of meat preparation was collected.

†Doneness levels: rare or medium, scores 3–4; mostly well-done, score 5; consistently well-done or very well-done, scores 6–9.

‡Adjusted for age, total energy intake, family history of breast cancer, hormone replacement therapy, and waist-to-hip ratio.

hydrocarbons, formed during high-temperature cooking of animal foods may be related to the risk of breast cancer.

Heterocyclic amine exposure, as measured indirectly in epidemiologic studies by levels of fried-food intake and a preference for heavily browned (well-done) meat, has been repeatedly shown to be related to an increased risk of colorectal cancer *(30,31)*. Although many epidemiologic studies have been conducted to evaluate dietary hypotheses for breast cancer, only a few of them have investigated the relationship between the intake of well-done meat and the risk of breast cancer. In a prospective cohort study in Finland *(15)*, a significant 80% increase in risk of breast cancer was found for high intake of fried meats, while intake of non-fried meat was not related to risk. High intake of fried meat was also reported to be positively associated with breast cancer in a recently published hospital-based, case–control study *(19)*. In that study, a 2.7-fold elevated risk (95% CI = 1.61–4.55) of breast cancer was observed among women in the uppermost quartile of fried meat intake, whereas intake of boiled meat was inversely related to breast cancer risk. These studies suggest that the risk of breast cancer may be more closely related to meat-cooking methods than to the level of meat intake per se. A positive relationship between intake of fried meat and breast cancer was also reported from several early case–control studies *(16–18)*. As with the two more recent studies *(15,19)*, however, information on other measures of heterocyclic amine exposures, such as degree of meat doneness and intake of grilled, barbecued, or broiled meats, was usually not obtained. Therefore, considerable misclassification in the assessment of heterocyclic amine exposure may exist in these previous studies.

In contrast to the strong positive association of doneness levels of meat with breast cancer, we found only a weak positive association for intake of fried, grilled, and broiled meats. It is likely that an individual's intake preference for meat doneness level is more consistent over time than preference for cooking method, especially when one considers all settings (e.g., restaurant or home) where a person consumes food. Thus, recall of doneness level may be more accurate than for preparation type. It is also possible that the use of photographs that represented various doneness levels of meats facilitated the ascertainment of information for this set of variables. Alternatively, doneness level may be a stronger predictor for cancer risk than intake levels of fried, grilled, and broiled meat with all doneness levels combined. Indeed, it has been shown that meats that have been fried, grilled, or broiled to ''just until done'' contain very low levels of heterocyclic amines *(21)*. Therefore, it is important in epidemiologic studies to obtain information on the level of meat doneness in the

assessment of heterocyclic amine exposure.

As with any epidemiologic study of diet and cancer, the measurement error in assessing dietary intake may be a concern in this study, particularly because the questionnaire used in this study was relatively new. By use of a similar questionnaire, well-done meat was found to increase the risk of colorectal adenomas in a recent study *(32)*, providing some assurance to the validity of this questionnaire. Furthermore, there is no reason to speculate that breast cancer case subjects would differentially recall intake of well-done to very well-done meats versus control subjects, particularly because the hypothesis investigated in this study was relatively new, and consumption of well-done hamburger is recommended in news media to reduce the risk of *Escherichia coli* infection. Therefore, the measurement errors in dietary assessment are most likely to be random, which may attenuate the exposure–disease association in most situations. We have adjusted for all other risk factors for breast cancer in the analyses; however, residual confounding, particularly from other dietary variables that may have been measured with error, may remain a concern. The participation rate (60%–75%) in this study was comparable to most case–control studies. Still, about 40% of case subjects and 25% of control subjects did not participate, and this may have caused selection bias if participation were related to exposure. As shown in Table 1, however, participants were, in general, similar to the subcohort of women who were eligible for the study in virtually all baseline risk factors and dietary habits, indicating that the potential for a selection bias may be minimal.

In summary, this case–control study found that intake preference for well-done meats and high intake of well-done, particularly very well-done, meats were associated with an increased risk of breast cancer. These findings, along with evidence accumulated from previous epidemiologic and laboratory studies, strongly suggest that heterocyclic amines and possibly other compounds formed during high-temperature cooking may be breast carcinogens in humans. It has been shown that several metabolic enzymes are involved in the activation or detoxification of heterocyclic amines in humans *(33,34)*, and investigation into the associations of these enzymes with breast cancer risk may help shed light on causal pathways for this common cancer. Therefore, future epidemiologic studies exploring the heterocyclic amine–breast cancer hypothesis should focus on the improvement of exposure assessment of heterocyclic amines and evaluate the potential effect of genetic variability in metabolic activities of these enzymes in the association of heterocyclic amine exposure and breast cancer risk.

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NOTES

[1]*Editor's note:* SEER is a set of geographically defined, population-based central tumor registries in the United States, operated by local nonprofit organizations under contract to the National Cancer Institute (NCI). Each registry annually submits its cases to the NCI on a computer tape. These computer tapes are then edited by the NCI and made available for analysis.

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Relationship Between Number of Ovulatory Cycles and Accumulation of Mutant p53 in Epithelial Ovarian Cancer

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Background: **It has been suggested that increased numbers of ovulations might increase the risk of p53 gene (also known as TP53) mutation in the ovarian epithelium, thereby leading to the development of cancer. The data supporting this hypothesis have come from an observation that accumulation of p53 protein in epithelial ovarian cancer was strongly associated with increasing numbers of ovulatory cycles. We have further investigated the association between ovulatory history and p53 gene mutation by use of data from a large case–control study of ovarian cancer in Australia.** ***Methods:*** **Tissue blocks were available for immunohistochemical analysis of p53 protein from 234 case subjects, aged 18–79 years, who had invasive epithelial ovarian cancer. Epidemiologic data were also available for these women and for 855 control subjects. Case–case comparisons were made by use of prevalence ratios and 95% confidence intervals (CIs), and case–control comparisons were made by use of odds ratios (ORs) and 95% CIs. All statistical tests were two-sided.** ***Results:*** **There was no association between p53 accumulation and years of ovulation. Women with p53-positive cancers had undergone an average of 29.3 years of ovulation compared with 29.0 years of ovulation for women with p53-negative cancers (*P* = .8). Although the overall risk of ovarian cancer development was significantly increased in women who had undergone more years of ovulation (OR = 2.17; 95% CI = 1.54–3.05—for ⩾35 years versus <23 years of ovulation), there was no difference in the risk associated with p53-positive and p53-negative cancers.** ***Conclusions:*** **These results confirm the association between increased ovulation and ovarian cancer risk but do not support the hypothesis that this association is due to an increased risk of p53 mutation with a greater number of ovulatory cycles. [J Natl Cancer Inst 1998;90:1729–34]**

There is evidence that the repeated process of ovulation plays a role in the development of ovarian cancer—the ''incessant ovulation'' theory *(1)*. This theory is supported by epidemiologic data showing that factors that suppress ovulation are consistently associated with reduced risks of ovarian cancer *(2)*. The mechanism that underlies this process is, however, unclear. The most frequently observed molecular alteration in ovarian tumors is mutation of the p53 (also known as TP53) tumor-suppressor gene, which leads to production of abnormal p53 protein that has an extended half-life and thus accumulates in the cells. Mutation and/or accumulation of p53 have been reported in about 50% of ovarian cancers *(3)*. A majority of these p53 mutations have been shown to be transitions *(4)*, the type of mutation that arises most commonly as a result of spontaneous errors in DNA synthesis during cell proliferation *(5)*. Ovulation is associated with damage and repair of the ovarian epithelium, and it has been suggested that these periods of increased epithelial cell proliferation could increase the risk of p53 mutation *(6)*. Mutations would, therefore, be expected to occur more commonly in women who had undergone a greater number of ovulatory cycles, leading to the development of an etiologically distinct subgroup of p53-positive tumors in these women.

Data to support this hypothesis have come from a recent report *(7)* that accumulation of mutant p53 in epithelial ovarian cancer was strongly associated with

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See ''Notes'' following ''References.''

an increasing number of ovulatory cycles. Compared with women who had undergone fewer ovulatory cycles, women who had undergone moderate or high numbers of ovulatory cycles were significantly more likely to have a p53-positive tumor with odds ratios (ORs) of 7.7 (95% confidence interval [CI] = 1.6–37.8) and 7.8 (95% CI = 1.3–47.6), respectively. As a result, in case–control analyses, an increasing number of ovulations was a strong risk factor for p53-positive cancer but was not related to p53-negative cancer. It has been suggested, however, that the fact that the women with p53-positive cancers were older than those with p53-negative cancers could potentially explain the observed association between p53 and the number of ovulatory cycles *(8)*. The original authors have since shown that, when p53-positive case subjects were age matched to control subjects, the association between number of ovulations and p53-positive cancer was strengthened *(9)*. It is still possible, however, that the apparent difference between p53-positive and p53-negative case subjects could be due to confounding, perhaps by age.

The possibility of a link between repeated ovulation, p53 mutation, and the development of ovarian cancer is intriguing. We have, therefore, further investigated the association between ovulation, p53, and ovarian cancer, utilizing data from a subset of women with invasive epithelial ovarian cancer who took part in a large case–control study of ovarian cancer in Australia.

Subjects and Methods

Study Population

Case subjects were women with histologically confirmed invasive epithelial ovarian cancer and control women who had participated in a large case–control study *(10)*. For the original study, case subjects, aged 18–79 years, who were diagnosed during the period 1991 through 1992 and who were registered in the major gynecology–oncology treatment centers in the three most populous Australian states (i.e., New South Wales, Victoria, and Queensland), were ascertained. In Queensland, case subjects diagnosed during August to December 1990 and throughout 1993 were also included. Of 915 eligible case subjects, 824 (90%) were interviewed; of those interviewed, 677 (82% of interviewed subjects, 74% of all ascertained case subjects) had invasive cancer and thus were eligible for inclusion in this study. Control subjects were chosen at random from the electoral roll (enrollment to vote is compulsory in Australia) to give an age and geographic distribution similar to that expected for the case subjects. Of 1178 eligible control subjects, 855 (73%) were interviewed.

Data Collection and Analysis

All case and control subjects had been interviewed by trained interviewers using standard techniques *(10)*. Sociodemographic information, including level of education, usual body mass index (BMI, kg/m^2), and family history of cancer, was obtained as well as detailed reproductive and contraceptive histories. These data were collected by use of a monthly calendar, completed by the interviewer, to record salient events in a woman's life from ages 15 to 50 years, including age at menarche, menstrual cycle length, parity, history of breast feeding, menopausal status, history of hysterectomy or tubal ligation, and use of the oral contraceptive pill and hormone replacement therapy. We calculated a woman's potential ovulatory life by subtracting age at menarche from age at menopause in the case of postmenopausal women (or age at diagnosis or age at interview for premenopausal case subjects and control subjects, respectively). We also subtracted

Table 1. Comparison of case subjects with and without material available for p53 immunohistochemical analysis

	Case subjects with material available (n = 234)	Case subjects without material available (n = 443)	*P**
Personal characteristics			
Mean (standard deviation) age at diagnosis, y	57.7 (11.7)	57.7 (12.3)	.97
Mean (standard deviation) age at menarche, y	13.6 (5.8)	13.1 (1.6)	.2
Mean (standard deviation) y of ovulation	29.2 (9.1)	28.6 (9.1)	.4
Educated beyond high school, %	47.8	38.0	.01
Usual body mass index, %			
<20.44 kg/m^2	13.0	12.6	.9
20.44–22.31 kg/m^2	20.9	19.6	
22.32–25.21 kg/m^2	29.6	26.9	
25.22–28.80 kg/m^2	20.0	22.0	
>28.80 kg/m^2	16.5	18.9	
Postmenopausal, %	67.4	67.5	.98
Duration of oral contraceptive pill use, %			
Never	55.0	54.7	.3
<24 mo	15.3	13.2	
24–59 mo	9.6	11.6	
60–119 mo	14.4	11.1	
⩾120 mo	5.7	9.4	
Hysterectomy, %	13.3	15.6	.4
Ever used HRT,† %	29.9	23.3	.06
Ever breast-fed, %	68.4	61.4	.07
Parity, %			
0	21.8	20.8	.7
1	12.4	14.2	
2	26.9	30.5	
3	18.8	18.7	
4	10.7	8.4	
⩾5	9.4	7.5	
Ever smoked, %	37.2	41.5	.3
Tumor characteristics			
Histology,‡ No. (%)			
Serous	144 (61.5)	225 (51.0)	.005
Endometrioid or clear cell	47 (20.1)	111 (25.2)	
Mucinous	14 (6.0)	39 (8.8)	
Undifferentiated	10 (4.3)	44 (10.0)	
Mixed histology	19 (8.1)	22 (5.0)	
Stage§			
I	40 (17.1)	89 (20.1)	.002
II	18 (7.7)	41 (9.3)	
III	131 (56.0)	180 (40.6)	
IV	15 (6.4)	32 (7.2)	
Unknown	30 (12.8)	101 (22.8)	

*Two-sided *P* values from Student's *t* test for continuous variables and χ^2 test for categorical variables.

†HRT = hormone replacement therapy.

‡Information on histologic type was missing for two case subjects who did not have material available for p53 immunohistochemistry.

§*See* reference *(13)*.

periods of time when a woman was not ovulating because she was using the oral contraceptive pill, pregnant (full-term and aborted pregnancies), or amenorrheic for some other reason (including periods of breast-feeding). We divided the number of months of ovulation by the woman's menstrual cycle length in months to calculate her total number of ovulations, and we then divided this total by 13, the average number of menstrual cycles per year, to create the variable ''ovulation years.''

Immunohistochemistry

Paraffin sections (4 μm) were dewaxed and rehydrated to distilled water through descending graded alcohols, then transferred to Tris-buffered saline (TBS; 0.05 *M* Tris in 0.15 *M* NaCl) at pH 7.4. The sections were subjected to microwave antigen retrieval in 0.01 *M* citric acid (pH 6.0) as described by Shi et al. *(11).* Endogenous peroxidase activity was quenched by incubation of the sections in 0.3% H_2O_2 and 0.1% sodium azide in TBS for 10 minutes. After being thoroughly washed in TBS, the sections were immersed in 4% commercial non-fat skim milk powder in TBS for 15 minutes to inhibit nonspecific antibody binding, after which they were transferred to a humidified chamber and covered with 10% normal (nonimmune) goat serum for 30 minutes. Excess serum was decanted from the sections, and the primary monoclonal antibody (murine anti-p53, clone DO7; Novocastra Laboratories, Ltd., Newcastle-upon-Tyne, U.K.) diluted 1 : 100 in TBS was applied. The sections were incubated with primary antibody overnight at room temperature. After this and subsequent incubations, the sections were washed thoroughly in three changes of TBS for 5 minutes each, the first wash containing 0.5% Triton X-100 to reduce nonspecific antibody binding. Sections were then incubated for 30 minutes with prediluted biotinylated goat anti-mouse immunoglobulins (Zymed Laboratories, Inc., San Francisco, CA) and then with prediluted streptavidin–horseradish peroxidase conjugate (Zymed Laboratories, Inc.) for 15 minutes. Antigenic sites were revealed by incubation of the sections in 0.05% 3,3′-diaminobenzidine in TBS with H_2O_2 as substrate. After being washed in gently running tap water, the sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted with DePeX (BDH Laboratory Supplies, Poole, U.K.). Sections of human breast cancers previously demonstrated to stain positively for p53 were run with each batch of immunohistochemical staining to act as positive controls. As negative controls, serial sections of each tumor were stained as described above, omitting the primary antibody.

By use of the same methodology, a random sample of 25 specimens was also stained for p53 accumulation using another primary antibody, clone PAb1801 (Dako Australia, Sydney, Australia).

One histopathologist (M. C. Cummings) scored the immunohistochemical staining. Note was made of the proportion of positive tumor cells (0%, 1%–10%, 11%–25%, 26%–50%, 51%–75%, or 76%–100%) present on each slide. Wild-type p53 protein has a very short half-life and is thus rarely detected in normal tissues. Mutation of the p53 gene produces an abnormal p53 protein with an extended half-life leading to accumulation of the protein in the cell. Therefore, if more than 10% of cells on a slide stained positively for p53, the tumor was classified as p53 positive *(7).*

Statistical Analysis

Case–case comparisons were made by the calculation of the ratio of the prevalence of p53 accumulation among case subjects at each level of the explanatory variable relative to the prevalence in the referent category. Prevalence ratios (PRs) and 95% CIs for different strata of a given variable were calculated using PROC PHREG in SAS *(12),* adjusting for age group and histologic type of cancer by use of a separate indicator term to represent each category. Associations with p53 accumulation were evaluated with the use of Student's *t* test for continuous variables and χ^2 tests for categorical variables. For case–control analyses, results are presented as ORs and 95% CIs calculated with the use of PROC LOGISTIC in SAS to adjust for age group, menopausal status, and parity. For all analyses, the referent category was defined as the unexposed or lowest group. For education level, exposure was defined as a lack of higher education; for menopausal status, postmenopausal women were considered exposed. All statistical tests were two-sided.

RESULTS

Of the 677 women with invasive ovarian cancer who participated in the original case–control study, material was available from 234 (35%) for p53 immunohistochemical analysis. Availability varied substantially by state; material was available from 51% of 262 case subjects from Queensland, from 34% of 191 case subjects from New South Wales, and from 16% of 224 case subjects from Victoria ($P<.0001$). Availability also varied depending on the histology and stage of the tumors (Table 1).

Case subjects with material available did not differ significantly from case subjects without material available with respect to their age at diagnosis, years of ovulation, age at menarche, usual body mass index, menopausal status, use of the oral contraceptive pill, parity, smoking, or history of hysterectomy (Table 1). There were minor differences between the groups with respect to their history of breast-feeding (68.4% of case subjects with tissue available versus 61.4% of case subjects with no tissue available had breast-fed; $P = .07$) and use of hormone replacement therapy (29.9% versus 23.3%; $P = .06$). The only statistically significant difference was with respect to the proportion of women who had received an education beyond high school (47.8% versus 38.0%; $P = .01$).

A total of 152 (65.0%) of the 234 tumors stained positively for p53 protein. The proportion of tumors with different histologies that stained positively varied substantially, however, being 69.4% among serous cancers and 72.3% among endometrioid or clear cell cancers but only 36.8%, 42.9%, and 50.0% among mixed histology, mucinous, and undifferentiated tumors, respectively (Table 2). There was little variation by stage of tumor *(13),* although the small group of stage IV tumors (13 of 15) was slightly more likely to stain positively for p53 than the group of stage I, II, and III tumors combined (119 of 189) (86.7% versus 63.0%; $P = .06$).

Table 3 shows the association between p53 protein accumulation and a range of sociodemographic and reproductive variables. Results are expressed as the percentage of tumors in each group that

Table 2. Number and percent of tumors positive for p53 by characteristics of the ovarian tumors from case subjects with material available

	Total No. of case subjects	p53-positive tumors		χ^2 (two-sided *P*)
		No.	%	
Histology				
Serous	144	100	69.4	13.0
Endometrioid or clear cell	47	34	72.3	(.01)
Mucinous	14	6	42.9	
Undifferentiated	10	5	50.0	
Mixed histology	19	7	36.8	
Stage*				
I	40	24	60.0	3.8
II	18	12	66.7	(.4)
III	131	83	63.4	
IV	15	13	86.7	
Unknown	30	20	66.7	

*In stages I and II, the tumors are localized to the peritoneum, whereas in stages III and IV, the tumors are metastatic. *See* reference *(13).*

Table 3. Number and percent of ovarian tumors positive for p53 stratified by characteristics of the women, with adjusted prevalence ratios and 95% confidence intervals

Characteristic	Total No. of case subjects*	p53-positive		Prevalence ratio†	95% confidence interval
		No.	%		
Age group, y					
<45	33	18	54.5	1.0	Referent
45–54	59	40	67.8	1.12	0.63–1.98
55–64	69	46	66.7	1.14	0.65–2.01
⩾65	73	48	65.8	1.16	0.67–2.03
Age at menarche, y					
<12	30	18	60.0	1.0	Referent
12–13	106	68	64.2	1.06	0.63–1.79
⩾14	96	65	67.7	1.12	0.66–1.91
Educated beyond high school					
Yes	111	76	68.5	1.0	Referent
No	121	74	61.2	0.90	0.64–1.27
Family history of ovarian cancer					
No	225	145	64.4	1.0	Referent
Yes	9	7	77.8	1.12	0.73–1.71
Usual body mass index, kg/m^2					
<20.44	30	18	60.0	1.0	Referent
20.44–22.31	48	30	62.5	0.98	0.54–1.77
22.32–25.21	68	44	64.7	1.00	0.57–1.74
25.22–28.80	46	32	69.6	1.08	0.60–1.95
>28.80	38	25	65.8	1.07	0.58–1.97
Menopausal status					
Premenopausal	66	38	57.6	1.0	Referent
Postmenopausal	168	114	67.9	1.27	0.70–2.33
Duration of oral contraceptive pill use, mo					
Never	126	83	65.9	1.0	Referent
<24	35	25	71.4	1.14	0.72–1.81
24–59	22	12	54.5	0.86	0.46–1.61
60–119	33	19	57.6	0.90	0.53–1.52
⩾120	13	11	84.6	1.35	0.70–2.59
Ever breast-fed					
No	74	42	56.8	1.0	Referent
Yes	160	110	68.8	1.24	0.86–1.78
Parity					
0	51	32	62.7	1.0	Referent
1	29	18	62.1	1.00	0.56–1.78
2	63	43	68.3	1.12	0.70–1.78
3	44	29	65.9	1.15	0.69–1.91
4	25	13	52.0	0.82	0.43–1.56
⩾5	22	17	77.3	1.16	0.64–2.11
Ovulation, y					
<23	58	36	62.1	1.0	Referent
23–29.9	61	41	67.2	1.02	0.64–1.62
30–34.9	55	37	67.3	0.98	0.60–1.63
⩾35	60	38	63.3	1.00	0.60–1.65
Ever smoked					
No	147	94	64.0	1.0	Referent
Yes	87	58	66.7	1.04	0.74–1.45

*Totals may vary because of missing data.
†Adjusted for age group and histology of tumor.

stained positively for p53, together with PRs and 95% CIs. There was no suggestion that p53-positive tumors were more common in women who had undergone more ovulatory cycles. Women with p53-positive tumors had undergone an average of 29.3 years of ovulation compared with 29.0 years among women with p53-negative tumors ($P = .8$). Reclassification of the women by use of the same cut points as those used by Schildkraut et al. *(7)* (⩽234, 235–375, 376–533, and >533 ovulatory cycles, equivalent to ⩽18, 18.1–28.8, 28.9–41, and >41 ovulation years) resulted in groups of very unequal sizes because of the older age of the women in our study. With the use of these cut points, however, there was still no association between the number of ovulatory cycles and p53 status (adjusted PR [95% CI] = 1.0 [referent], 1.04 [0.56–1.92], 0.99 [0.53–1.88], and 0.97 [0.42–2.25], respectively).

Case–control comparisons showed that case subjects with both p53-positive and p53-negative tumors had undergone more ovulatory cycles than control subjects (Table 4). However, the association was, if anything, slightly stronger for p53-negative case subjects. When premenopausal and postmenopausal women were considered separately, the association between number of ovulatory cycles and ovarian cancer was stronger for premenopausal women.

The prevalence of p53-positive tumors did not vary significantly with any of the other variables studied (Table 3), although p53 positivity was slightly more common among women who were older at menarche, who had breast-fed, who had the greatest number of children, and who had taken the oral contraceptive pill the longest. These are all variables that would reduce their total number of ovulations. Women aged less than 45 years were slightly less likely to have a p53-positive tumor; as a result, women with p53-positive tumors were, on average, 1.8 years older than women with p53-negative tumors (58.3 years versus 56.5 years; $P = .3$). Tumors that were p53 positive were no more common in women with a family history of ovarian cancer than in other women.

DISCUSSION

These results confirm an association between increasing numbers of ovulations and ovarian cancer, but they do not support the hypothesis that incessant ovulation leads to p53 mutation and hence to cancer. The overall association between number of ovulations and cancer risk is consistent with the results reported by Schildkraut et al. *(7)* for p53-positive cancer, with the lower ORs in the present study reflecting the greater proportion of postmenopausal women in the study group *(2)*. The lack of an association between p53 accumulation and the number of ovulations is, however, contrary to the results previously reported by Schildkraut et al. *(7)*, and several possible explanations for this difference were explored.

It is likely that there is some misclassification of tumors with respect to their p53 status. In particular, not all p53 mutations will lead to the accumulation of a mutant p53 gene product, and these mu-

Table 4. Comparison of case subjects with invasive ovarian cancer and control subjects with respect to numbers of ovulation years

Ovulation years	No. of case subjects	No. of control subjects	Odds ratio*	95% confidence interval
All invasive tumors				
<23 y	172	348	1.0	Referent
23–29.9 y	175	210	1.57	1.17–2.12
30–34.9 y	155	161	1.74	1.25–2.42
⩾35 y	175	136	2.17	1.54–3.05
p53-positive tumors				
<23 y	36	348	1.0	Referent
23–29.9 y	41	210	1.70	1.00–2.88
30–34.9 y	37	161	2.25	1.14–3.58
⩾35 y	38	136	2.03	1.25–4.05
p53-negative tumors				
<23 y	22	348	1.0	Referent
23–29.9 y	20	210	1.70	0.83–3.49
30–34.9 y	18	161	2.06	0.95–4.50
⩾35 y	22	136	2.69	1.24–5.84
Premenopausal women				
<23 y	96	219	1.0	Referent
23–29.9 y	60	67	1.93	1.20–3.09
30–34.9 y	26	15	3.56	1.66–7.63
⩾35 y	21	8	5.21	2.08–13.0
Postmenopausal women				
<23 y	76	129	1.0	Referent
23–29.9 y	115	143	1.28	0.88–1.88
30–34.9 y	129	146	1.39	0.95–2.04
⩾35 y	154	128	1.86	1.26–2.73

*Odds ratios were adjusted for age group (<45, 45–54, 55–64, and ⩾65 years), menopausal status, and parity.

tations would not be detected by immunohistochemical analysis. For our study to show no association between the number of ovulatory cycles and p53 in the presence of a true association as strong as that reported by Schildkraut et al. *(7)*, the p53 staining would have to be essentially random. The antibody used for the p53 immunohistochemical analysis in our study (clone DO7) was not the same as that used by Schildkraut et al. (clone PAb1801). However when 25 samples were selected at random and retested by use of the antibody clone PAb1801, 24 of them (96%; 10 p53-negative and 14 p53-positive) were classified identically with the use of the two antibodies. This result indicates that there are no major differences between the two staining methods, and this degree of misclassification could not explain the complete lack of association in our study.

There were also differences between the women included in the two studies. The women in our study were, on average, about 12 years older than those in the study reported by Schildkraut et al. *(7);* therefore, a greater proportion of the women in our study were postmenopausal. In addition, 144 (61.5%) of the 234 case subjects included in our study had serous cancers and 47 (20.1%) had endometrioid or clear cell cancers compared with only 44% serous cancers and 37% endometrioid or clear cell cancers in the study by Schildkraut et al. Our study group included 92 women aged less than 55 years, the maximum age included in the study by Schildkraut et al. The mean age of these women (45.9 years; standard deviation = 6.7) was identical to that in the study by Schildkraut et al. (45.9 years; standard deviation = 7.5). Restricting the analyses to these women had no effect on the results of our study, with PRs of 1.2 for women with 23.0–29.9 years of ovulation, 1.0 for those with 30.0–34.9 years of ovulation, and 1.0 for those with 35 years or more years of ovulation compared with women with fewer than 23 years of ovulation. Similarly, when premenopausal and postmenopausal case subjects were considered separately, there was no association between p53 accumulation and number of ovulations in either group. The percentage of serous cancers that were p53 positive was similar in the two studies (69.4% in our study versus 64.0% in the study by Schildkraut et al.); however, in our study, endometrioid and clear cell cancers were more likely to be p53 positive than in the study by Schildkraut et al. (72.3% versus 48.6%). There was no association between p53 accumulation and number of ovulations among any of the main subtypes in our study. None of these differences between the study populations could, therefore, explain the different results from the two studies.

A further difference between the two studies was the adjustment made for cycle length in the calculation of number of ovulations in our study. The data were reanalyzed excluding this adjustment, but this exclusion had no effect on the results.

It has been suggested that the strong association between the number of ovulatory cycles and p53 accumulation reported by Schildkraut et al. *(7)* might be explained by the fact that their p53-positive case subjects were, on average, 3 years older than the p53-negative case subjects *(8)*. In our study, p53-positive case subjects were, on average, 1.8 years older than the p53-negative case subjects. If p53-positive case subjects had undergone more ovulations simply because they were older, this confounding could potentially have created a spurious association between p53 accumulation and the number of ovulations, but it could not have masked a real association in this study. Furthermore, the lack of an association in our study does not simply represent a lack of statistical power to detect a significant difference between women with p53-positive and p53-negative cancers. The relative risks of p53-positive versus p53-negative cancer associated with increasing years of ovulation, as estimated by the PRs, are all 1.0 with 95% CIs from 0.6 to 1.6. This observation effectively rules out the possibility that the real differences could have been as great as those reported by Schildkraut et al. *(7)*, who observed a fourfold higher prevalence of p53-positive tumors (associated with adjusted ORs of 7.7–7.8) among women who had undergone greater numbers of ovulations.

Finally, tissue blocks were available for only one third of the eligible case subjects from the original study, and their availability varied by state and by cell type and stage of cancer. There was no difference in the prevalence of p53-positive cancers in the different states, so variation in availability by state is un-

likely to have biased the results. The proportion of tumors that stained positively for p53 did vary across cancers of different cell types, and variation in availability by type of cancer could, therefore, have affected the overall prevalence of p53-positive tumors within the study group. The women with material available for p53 staining did not, however, differ markedly from those from whom tissue blocks were not available with respect to any of the sociodemographic or reproductive variables studied. Although it is possible that there could be some other unmeasured differences, it is unlikely that these could be so great as to completely mask a true association as strong as that reported by Schildkraut et al. *(7)*.

The increased risk of ovarian cancer observed in our study in all women who had undergone the greatest numbers of ovulations is consistent with the hypothesis that incessant ovulation is associated with the development of ovarian cancer. Our results do not, however, support the suggestion that this association is due to an increased risk of p53 mutation in women who have undergone greater numbers of ovulatory cycles. Rather, they raise the possibility that the association between p53 accumulation and the number of ovulations observed by Schildkraut et al. *(7)* might have been an artifact, possibly resulting from the age difference between their p53-positive and p53-negative case subjects as has been suggested *(8)* or possibly resulting from some other unidentified confounder.

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NOTES

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Characterization of MOAT-C and MOAT-D, New Members of the MRP/cMOAT Subfamily of Transporter Proteins

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Background: **Multidrug resistance-associated protein (MRP) and canalicular multispecific organic anion transporter (cMOAT) are transporter proteins that pump organic anions across cellular membranes and have been linked to resistance to cytotoxic drugs. We previously identified MOAT-B, an MRP/cMOAT-related transporter, by use of a polymerase chain reaction approach. However, analysis of expressed sequence tag (EST) databases indicated that there might be additional MRP/cMOAT-related transporters. To further define the MRP/cMOAT subfamily of transporters, we used EST probes to isolate complementary DNAs for two related transporter proteins, MOAT-C and MOAT-D.** ***Methods:*** **MOAT-C and MOAT-D expression patterns in human tissues were determined by RNA blot analysis, and chromosomal localization of the genes was determined by fluorescence *in situ* hybridization.** ***Results:*** **MOAT-C is predicted to encode a 1437-amino-acid protein that, among eukaryotic transporters, is most closely related to MRP, cMOAT, and MOAT-B (about 36% identity). However, MOAT-C is less related to MRP and cMOAT than MRP and cMOAT are to each other (about 48% identity). Like MOAT-B, MOAT-C lacks an N-terminal membrane-spanning domain, indicating that the topology of this protein is similarly distinct from that of MRP and cMOAT. MOAT-D is predicted to encode a 1527-amino-acid protein that is the closest known relative of MRP (about 58% identity). MOAT-D is also highly related to cMOAT (about 47% identity). The presence of an N-terminal membrane-spanning domain indicates that the topology of MOAT-D is quite similar to that of MRP and cMOAT. MOAT-C transcripts are widely expressed in human tissues; however, MOAT-D transcript expression is more restricted. The MOAT-C and MOAT-D genes are located at chromosomes 3q27 and 17q21.3, respectively.** ***Conclusions:*** **On the basis of amino acid identity and protein topology, the MRP/cMOAT transporter subfamily falls into two groups; the first group consists of MRP, cMOAT, and MOAT-D, and the second group consists of MOAT-B and MOAT-C. [J Natl Cancer Inst 1998;90: 1735–41]**

Cellular resistance is a major obstacle to the successful use of anticancer chemotherapeutic agents. Studies of cell lines selected *in vitro* for resistance to natural product drugs suggest that at least two transporters of the adenosine triphosphate (ATP)-binding cassette (ABC) family represent important components of the cellular drug resistance machinery. P-glycoprotein (Pgp), the first transporter shown to confer natural product drug resistance, functions as an ATP-dependent pump that transports diverse lipophilic drugs across the plasma membrane *(1)*. The second transporter, multidrug resistance-associated protein (MRP), also confers resistance to natural product drugs *(2–4)*. Although Pgp and MRP confer similar resistance phenotypes *(5–7)*, their structures and substrate specificities are distinct. MRP shares only 23% amino acid identity with Pgp and, in contrast to Pgp, is capable of transporting organic anions such as glutathione *S*-conjugates *(8)*. More recently, isolation of canalicular multispecific organic anion transporter (cMOAT), a liver-specific MRP-related transporter, was reported *(9–11)*. Genetic and biochemical studies of rat strains that are deficient in cMOAT indicate that cMOAT is also an organic anion transporter *(10,11)*. Overexpression of the cMOAT transcript has been described in cisplatin-resistant cell lines *(9,12)*, and transfection of an antisense cMOAT construct was reported to confer enhanced sensitivity to cytotoxic drugs *(13)*. In addition, a cMOAT-deficient rat was reported to exhibit reduced biliary clearance of methotrexate *(14)*. These observations suggest that cMOAT may confer resistance to some cytotoxic drugs and that it may also be involved in the hepatobiliary excretion of anticancer agents.

The important functions of MRP and cMOAT led us to attempt the isolation of other related transporters. Using a degenerate polymerase chain reaction (PCR) approach, we previously isolated MOAT-B, an MRP/cMOAT-related transporter *(15)*. Analyses of expressed sequence tag (EST) database sequences in our laboratory (Belinsky MG, Kruh GD: unpublished data) and in other laboratories *(12,16)* suggest that additional human MRP-related transporters may exist, but their full-length coding sequences have not been reported.

The purpose of this study was to isolate the complementary DNAs (cDNAs) of two additional MRP/cMOAT subfamily members, MOAT-C and MOAT-D, and to compare their predicted amino acid sequences, topologies, and expression patterns with those of the known subfamily members, MRP, cMOAT, and MOAT-B. This comparison helps to define the MRP/cMOAT subfamily with regard to protein structure and tissue-specific expression.

Materials and Methods

Isolation of cDNAs of MOAT-C and MOAT-D

MOAT-C cDNA clones were isolated from bacteriophage libraries prepared from human ovarian cancer cell line A2780 and human leukemia cell line HL60 by plaque hybridization, with the use of Integrated Molecular Analysis of Genomes (I.M.A.G.E.) consortium cDNA clone 113196 as the initial probe *(17)*. The 5′ end of the MOAT-C-coding sequence was obtained by rapid amplification of cDNA ends (RACE) by use of a Marathon cDNA amplification kit (Clontech Laboratories, Inc., Palo Alto, CA) and cDNA prepared from a human ovarian cancer cell line. MOAT-D cDNA clones were isolated from bacteriophage libraries prepared from human liver and monocytes by use of the I.M.A.G.E consortium cDNA clone 208097 as the initial probe. Nucleotide sequence analysis was performed with an ABI 377 DNA sequencer, and sequences were assembled with the use of the Sequencher program (Gene Codes Corp., Ann Arbor, MI).

Protein sequence was predicted by use of the Wisconsin Genetics Computer Group Package version

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See ''Notes'' following ''References.''

9.1 (Madison, WI). To generate hydrophobicity plots, we used the Kyte–Doolittle algorithm with a window of seven residues. To generate amino acid sequence alignments, we used the PILEUP command (gap weight 3.0; length weight 0.1). Transmembrane-spanning segments, which are approximately 20-amino-acid stretches of nonpolar residues, were assigned by inspection and with the assistance of the TMAP program *(18)*. Nucleotide-binding folds, which are approximately 150- to 170-amino-acid hydrophilic domains characterized by the presence of Walker A (GXXG/AXGKS/T) and Walker B (ϕ_4D, where ϕ is a hydrophobic residue) motifs, and the signature C sequence (LSGGQ) of ABC transporters *(19)*, were identified in amino acid alignments with known ABC transporters. The accession numbers of proteins described in the text are as follows: MOAT-B, AF071202; MRP, P33527; cMOAT, U63970; YCF1 (i.e., yeast cadmium resistance factor 1), P39109; SUR (i.e., sulfonyl urea receptor), Q09428; CFTR (i.e., cystic fibrosis transmembrane conductance regulator), P13569; and MDR1, P08183.

RNA Blot Analysis

Blots containing poly A^+ RNA isolated from human tissues were purchased from Clontech Laboratories, Inc., and hybridized with MOAT-C, MOAT-D, or actin cDNA probes, according to the manufacturer's directions.

Chromosomal Localization

Preparation of metaphase spreads from phytohemagglutinin-stimulated lymphocytes of a healthy female donor and fluorescence *in situ* hybridization to chromosomes were carried out as previously described *(20)*. Segments of the MOAT-C or MOAT-D cDNAs inserted in plasmid pBluescript were biotinylated by nick translation in a reaction mixture containing 1 μg DNA, 20 μ*M* each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate, 1 μ*M* deoxythymidine triphosphate, 25 m*M* Tris–HCl buffer (pH 7.5), 5 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, 10 μ*M* biotin-16-deoxyuridine triphosphate (Boehringer Mannheim, Indianapolis, IN), 2 U DNA polymerase 1/deoxyribonuclease I (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD), and water to a total volume of 50 μL. The probe was denatured and hybridized overnight at 37 °C to chromosomes in the metaphase spreads. Hybridization sites were detected by use of fluorescein-labeled avidin (Oncor, Inc., Gaithersburg, MD) and amplified by addition of anti-avidin antibody (Oncor, Inc.) and a second layer of fluorescein-labeled avidin. The chromosome preparations were counterstained with diamidino-2-phenylindole (DAPI) and observed with a Zeiss Axiophot epifluorescence microscope equipped with a cooled charge coupled device camera (Photometrics, Tucson, AZ) operated by a Macintosh computer workstation. Digitized images of DAPI staining and fluorescein signals were captured, pseudo-colored, and merged by use of Oncor Image version 1.6 software.

RESULTS

Isolation of cDNAs of MOAT-C and MOAT-D

A BLAST search *(21)* of the EST database revealed two clones with significant similarity to MRP and cMOAT. The first clone (I.M.A.G.E. consortium clone 113196) was 1.2 kilobases (kb) in length, 800 base pairs (bp) of which encoded an MRP-related peptide. A segment of clone 113196 was used as a probe to screen bacteriophage libraries. The resulting cDNA clones contained 2 kb of additional coding sequence and 1 kb of additional 3′ untranslated sequence. An additional 1.7 kb of 5′ sequence was obtained by RACE, yielding a total of about 5.9 kb of cDNA. Nucleotide sequence analysis revealed an open reading frame of 4311 bp that was preceded by an in-frame stop codon located at position −93 and encoding a protein of 1437 residues, which we designated MOAT-C. A cDNA encoding a partial peptide of MOAT-C (residues 493–1438) was described recently *(22)*. The second sequence identified in our database search (I.M.A.G.E. consortium clone 208097) was 1.2 kb in length, of which 588 bp encoded an MRP-related peptide. A segment of this clone was used as the initial probe to screen bacteriophage libraries, and a total of about 5.2 kb of cDNA sequence was isolated. Nucleotide sequence analysis revealed an open reading frame of 4581 bp, which we designated MOAT-D. An upstream in frame stop codon was not present in the cDNA clones, and attempts to obtain additional upstream sequences by RACE were unsuccessful. The most upstream ATG, located at nucleotide position 6, was therefore designated as the putative translational initiation site.

Analysis of MOAT-C- and MOAT-D-Predicted Proteins

Typical features of ABC transporters were present in the predicted MOAT-C and MOAT-D proteins (Fig. 1, A; Fig. 1, B). The proteins were composed of hydrophobic domains containing potential transmembrane-spanning helices (α helical stretches of about 20 nonpolar residues) and two nucleotide-binding folds (conserved hydrophilic domains of about 150–170 amino acids that are present in all ABC family transporters). Conserved Walker A and B motifs, as well as conserved C motifs, the signature sequence of ABC transporters, were present in the nucleotide-binding folds. Computer-assisted analysis *(18)* of potential transmembrane-spanning helices of MOAT-C predicted 12 transmembrane helices with six helices in each of two membrane-spanning domains. This 6 + 6 configuration is in agreement with topological models proposed for several other ABC transporters *(23,24)* and is shown in Fig. 1, A. Alternative predictions of transmembrane segments were obtained with the use of different program parameters or input alignments. Comparison of the hydropathy profiles of MOAT-C with other MRP/cMOAT-related transporters (Fig. 2, A) indicated that its structure is similar to that of MOAT-B. Like MOAT-B, MOAT-C has two membrane-spanning domains, each of which is appended N-terminal to an ATP-binding fold. Neither MOAT-C nor MOAT-B has a hydrophobic extension of about 200 amino acids that is present in the organic anion transporters MRP, cMOAT, MOAT-D, and YCF1, as well as in the SUR, a related ABC transporters that is not an organic anion pump. MOAT-C is distinguished from MOAT-B, as well as from CFTR and MDR1, by the presence of an N-terminal hydrophilic extension of 88 amino acids.

In contrast to MOAT-B and MOAT-C, hydrophobicity analysis of MOAT-D indicated that it has three membrane-spanning domains (Fig. 1, B; Fig. 2, A). Similar to MRP, cMOAT, YCF1, and SUR, MOAT-D has an additional hydrophobic domain located at its N-terminus. A 5 + 6 + 6 configuration of transmembrane-spanning helices has been proposed for MRP, in which the N-terminal extension harbors five helices, and six helices are present in both the second and third membrane-spanning domains *(23,25–27)*. Inspection of an alignment of the MOAT-D and MRP amino acid sequences with the use of the GAP program indicated that proposed MRP transmembrane segments were conserved in MOAT-D. This 5 + 6 + 6 model for MOAT-D is shown in Fig. 1, B. An alternative configuration (5 + 6 + 4) was predicted by use of a computer-assisted analysis. MRP has been reported to have two N-linked glycosylation sites in its N-terminus (Asn-19 and Asn-23) and another site located between the first and second transmembrane-spanning helices of its third membrane-spanning domain (Asn-1006) *(26)*. Potential N-terminal (Asn-18) and distal N-glycosylation (Asn-1006/1007) sites were conserved in analogous positions in MOAT-D. Only

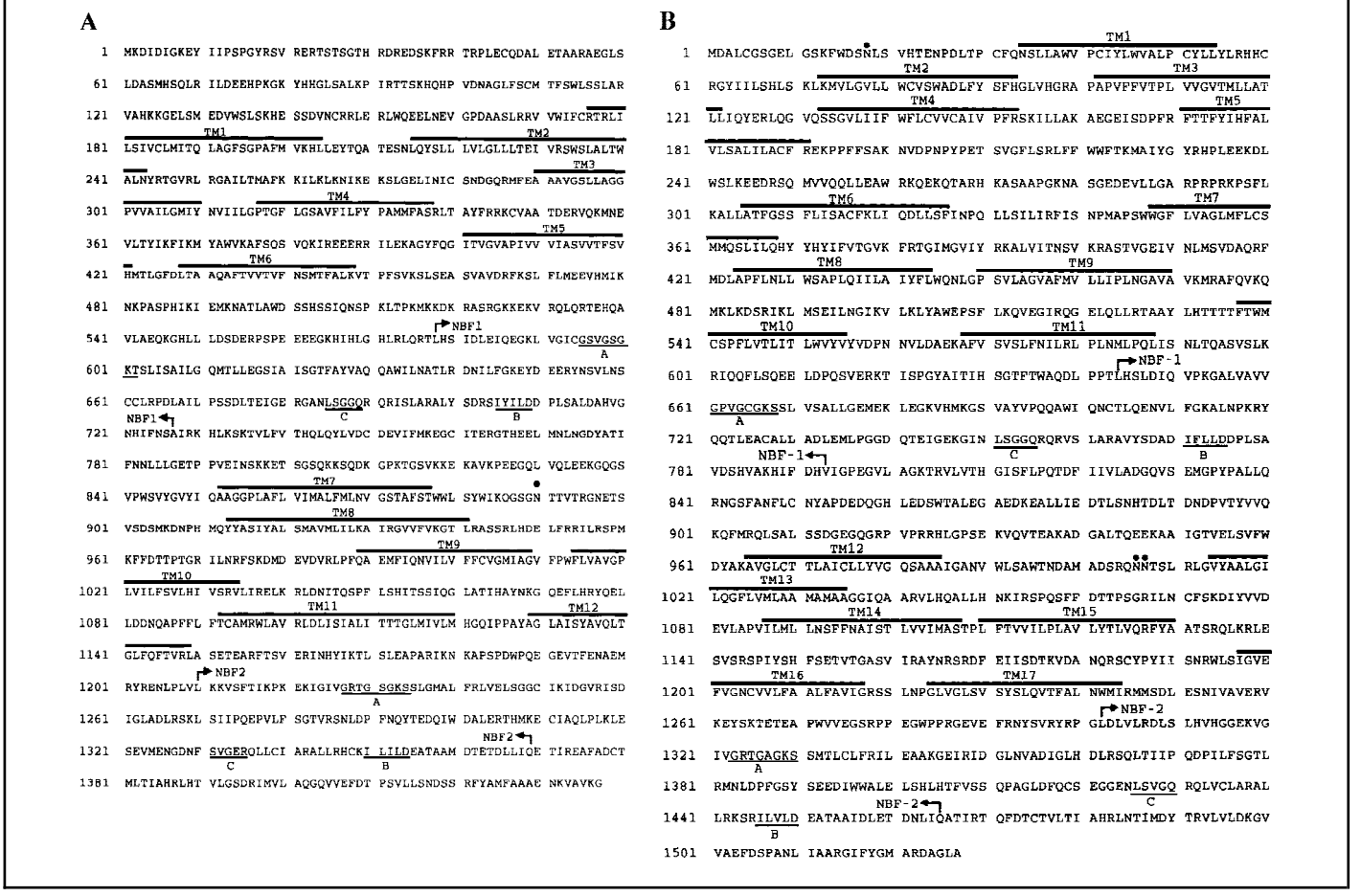

Fig. 1. Predicted structures of transporter proteins MOAT-C and MOAT-D. **A)** Structure of MOAT-C. **B)** Structure of MOAT-D. Numbered overbars indicate potential transmembrane (TM)-spanning helices (TM1–TM12 in MOAT-C and TM1–TM17 in MOAT-D). Horizontal arrows indicate the positions of nucleotide-binding folds (NBF) (amino-terminal = NBF1; carboxy-terminal = NBF2). Walker A and B motifs and the ABC transporter family signature sequence C are underlined. Bullets indicate the positions of potential N-glycosylation sites that are conserved with reported N-glycosylation sites in multidrug resistance-associated protein (MRP). The indicated MOAT-C transmembrane-spanning helices were predicted by use of the TMAP program and an input alignment of MOAT-B and MOAT-C. The indicated MOAT-D transmembrane helices are based on inspection of an alignment with MRP and hydropathy analysis with the use of the Kyte–Doolittle algorithm *(31)*.

the distal N-glycosylation site of MRP is potentially conserved in MOAT-C (Asn-890) and MOAT-B (Asn-746/754) *(15)*.

The degree of relatedness of the nucleotide-binding folds of ABC transporters is considered to be an indication of potential functional conservation. Comparison of the predicted amino acid sequences of the nucleotide-binding folds of MOAT-C and MOAT-D with other ABC transporters indicated that they were most closely related to those of the MRP/cMOAT subfamily transporters MRP, cMOAT, MOAT-B, and YCF1. As shown in Table 1, the nucleotide-binding fold 1 of MOAT-C was about equally related to MOAT-D, MRP, and cMOAT (57.3%–61.3% identity) and less similar to MOAT-B (49.3% identity). The nucleotide-binding fold 2 of MOAT-C shared about equal amino acid identity with these human transporters (56.9%–60.6% identity). Overall, MOAT-C was about equally related to the other five transporters, with 33.1%–36.5% amino acid identity. Aside from these transporters, MOAT-C was most closely related to SUR, with which its first and second nucleotide-binding folds shared about 49%/51% identity, and the CFTR, with which its nucleotide-binding folds shared about 44%/42% identity (data not shown in Table 1).

The nucleotide-binding folds of MOAT-D were clearly most closely related to those of MRP and cMOAT (67.3%–73.8%) but were slightly more related to those of MRP. In contrast, the nucleotide-binding folds of MOAT-D shared only 54.1%–57.3% identity with those of MOAT-C and MOAT-B. Overall, MOAT-D was again most closely related to MRP (57.6%) and cMOAT (46.8%), but it was substantially more related to MRP. Consistent with the analysis of nucleotide-binding folds, MOAT-D was much less related overall to MOAT-C and MOAT-B, with which it shared only 33.1% and 35.3% identity, respectively. An alignment (Fig. 2, B) of the amino acid sequences of the MOAT-C and MOAT-D nucleotide-binding folds with those of related transporters revealed that nucleotide-binding fold 1 of the MRP/cMOAT-related transporters is distinguished from that of SUR and MDR1 by the presence in the latter of 26 and 13 amino acid insertions, respectively. An insertion is also absent in CFTR.

Expression Pattern of MOAT-C and MOAT-D in Human Tissues

The tissue expression patterns of MOAT-C and MOAT-D were examined by RNA blot analysis. As shown in Fig. 3, A (upper panels), a MOAT-C transcript of

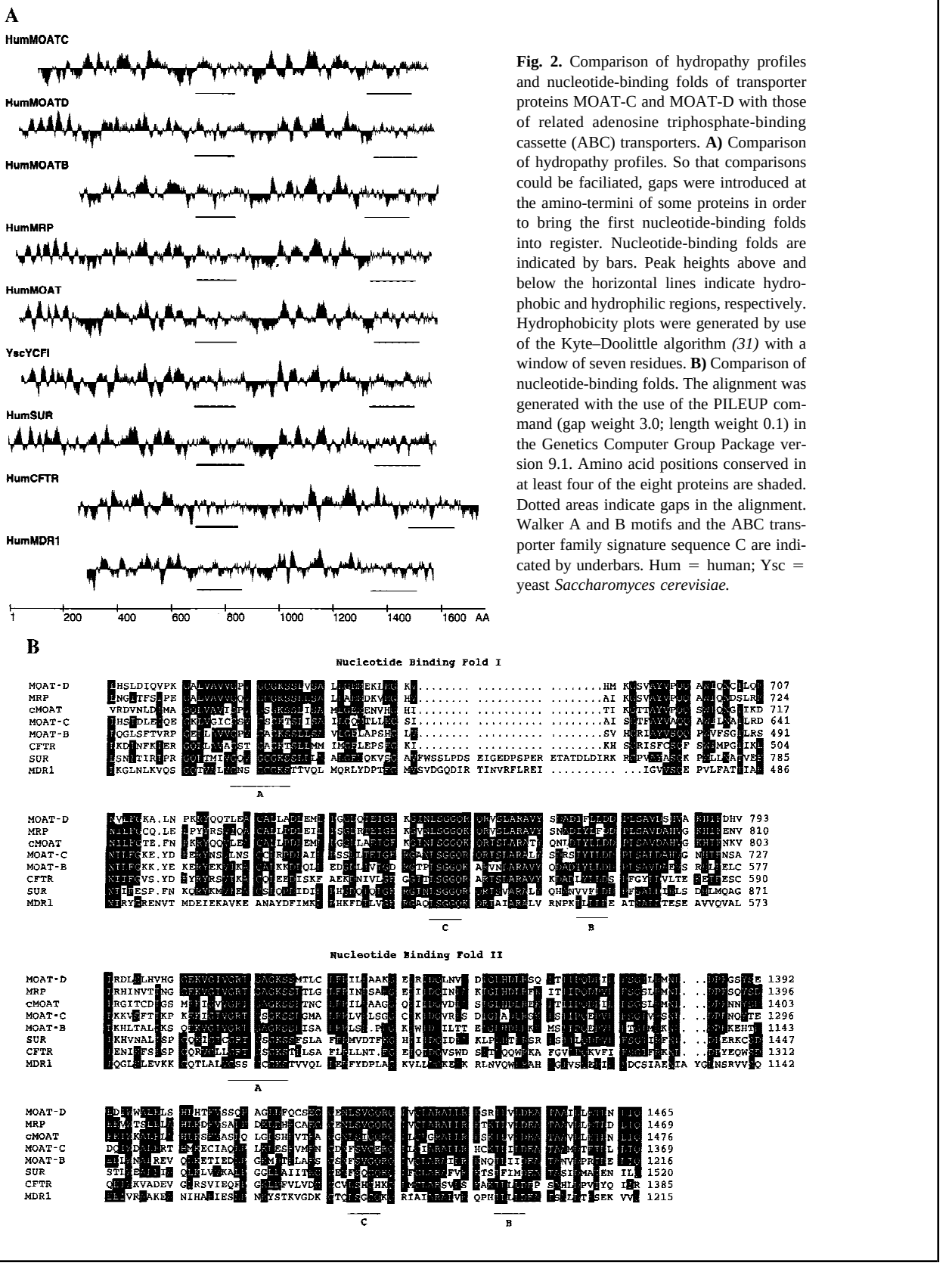

Fig. 2. Comparison of hydropathy profiles and nucleotide-binding folds of transporter proteins MOAT-C and MOAT-D with those of related adenosine triphosphate-binding cassette (ABC) transporters. **A)** Comparison of hydropathy profiles. So that comparisons could be faciliated, gaps were introduced at the amino-termini of some proteins in order to bring the first nucleotide-binding folds into register. Nucleotide-binding folds are indicated by bars. Peak heights above and below the horizontal lines indicate hydrophobic and hydrophilic regions, respectively. Hydrophobicity plots were generated by use of the Kyte–Doolittle algorithm *(31)* with a window of seven residues. **B)** Comparison of nucleotide-binding folds. The alignment was generated with the use of the PILEUP command (gap weight 3.0; length weight 0.1) in the Genetics Computer Group Package version 9.1. Amino acid positions conserved in at least four of the eight proteins are shaded. Dotted areas indicate gaps in the alignment. Walker A and B motifs and the ABC transporter family signature sequence C are indicated by underbars. Hum = human; Ysc = yeast *Saccharomyces cerevisiae.*

approximately 6.6 kb (at arrow) was readily detected in several tissues, with highest levels in skeletal muscle, intermediate levels in kidney, testis, heart, and brain, and low levels in most other tissues, including spleen, thymus, prostate, ovary, and placenta. Prolonged exposures were required for detection in lung and liver. MOAT-D (middle panels) was expressed as an approximately 6-kb transcript (at arrow). Compared with the expression pattern of MOAT-C,

Table 1. Amino acid identity among MRP/cMOAT subfamily members*

	% identity†					
	MOAT-C	MOAT-D	MOAT-B	MRP	cMOAT	YCF1
MOAT-C	—	33.1	36.5	35.8	36.2	33.6
	—	(57.3/56.9)	(49.3/59.1)	(60.0/59.4)	(61.3/60.6)	(46.7/58.8)
MOAT-D	33.1	—	35.3	57.6	46.8	38.1
	(57.3/56.9)	—	(55.3/54.1)	(70.7/73.8)	(67.3/70.0)	(52.7/61.3)
MOAT-B	36.5	35.3	—	39.4	36.8	38.8
	(49.3/59.1)	(55.3/54.1)	—	(57.3/61.6)	(53.3/55.3)	(56.0/57.2)
MRP	35.8	57.6	39.4	—	48.4	40.4
	(60.0/59.4)	(70.7/73.8)	(57.3/61.6)	—	(66.0/73.1)	(53.3/63.8)
cMOAT	36.2	46.8	36.8	48.4	—	38.8
	(61.3/60.6)	(67.3/70.0)	(53.3/55.3)	(66.0/73.1)	—	(50.7/61.9)
YCF1	33.6	38.1	38.8	40.4	38.8	—
	(46.7/58.8)	(52.7/61.3)	(56.0/57.2)	(53.3/63.8)	(50.7/61.9)	—

*cMOAT = canalicular multispecific organic anionic transporter; MRP = multidrug resistance-associated protein; YCF1 = yeast cadmium resistance factor 1.

†Values in columns = overall percent amino acid identity (percent identity of nucleotide-binding folds 1 and 2, NBF1/NBF2). Percent identity was obtained by use of the GAP command in the GCG package.

the MOAT-D expression pattern was more restricted, with high transcript levels in the colon, pancreas, liver, and kidney and lower levels in the small intestine, placenta, and prostate. Prolonged exposures were required to detect MOAT-D in the testis, thymus, spleen, and lung.

Chromosomal Localization of MOAT-C and MOAT-D Genes

The MOAT-C and MOAT-D gene localizations on chromosomes were determined by fluorescence *in situ* hybridization (Fig. 3, B). Hybridization of the MOAT-C probe to spreads of human lymphocytes in metaphase revealed specific labeling at human chromosome band 3q27 (upper panel). Fluorescent signals were detected on chromosome 3q in each of 22 metaphase spreads scored. Of 75 signals observed, 43 (57%) were on 3q. Paired (on sister chromatids) signals, which are indicative of the true target locus, were seen only at band 3q27. Hybridization of the MOAT-D probe revealed specific labeling at human chromosome band 17q21.3 (Fig. 3, B; lower panel). Fluorescent signals were detected on chromosome 17q in each of 21 metaphase spreads scored. Of 83 signals observed, 34 (41%) were at 17q21.3. Paired signals were seen only at band 17q21.3.

Discussion

The isolation of MOAT-C and MOAT-D cDNAs extends to five, the number of human MRP/cMOAT subfamily members for which full-length coding sequences are known. On the basis of the degree of amino acid similarity and overall topology, these proteins fall into two groups. The first group is composed of MOAT-D, MRP, and cMOAT. These three transporters are highly related, sharing about 47%–58% amino acid identity. MOAT-D is more closely related to MRP (about 58% identity) than is cMOAT to MRP (about 48% identity) and is thus the closest known relative of MRP. This group of transporters also has in common an N-terminal membrane-spanning domain that is predicted to harbor approximately five transmembrane helices. This N-terminal extension is also present in

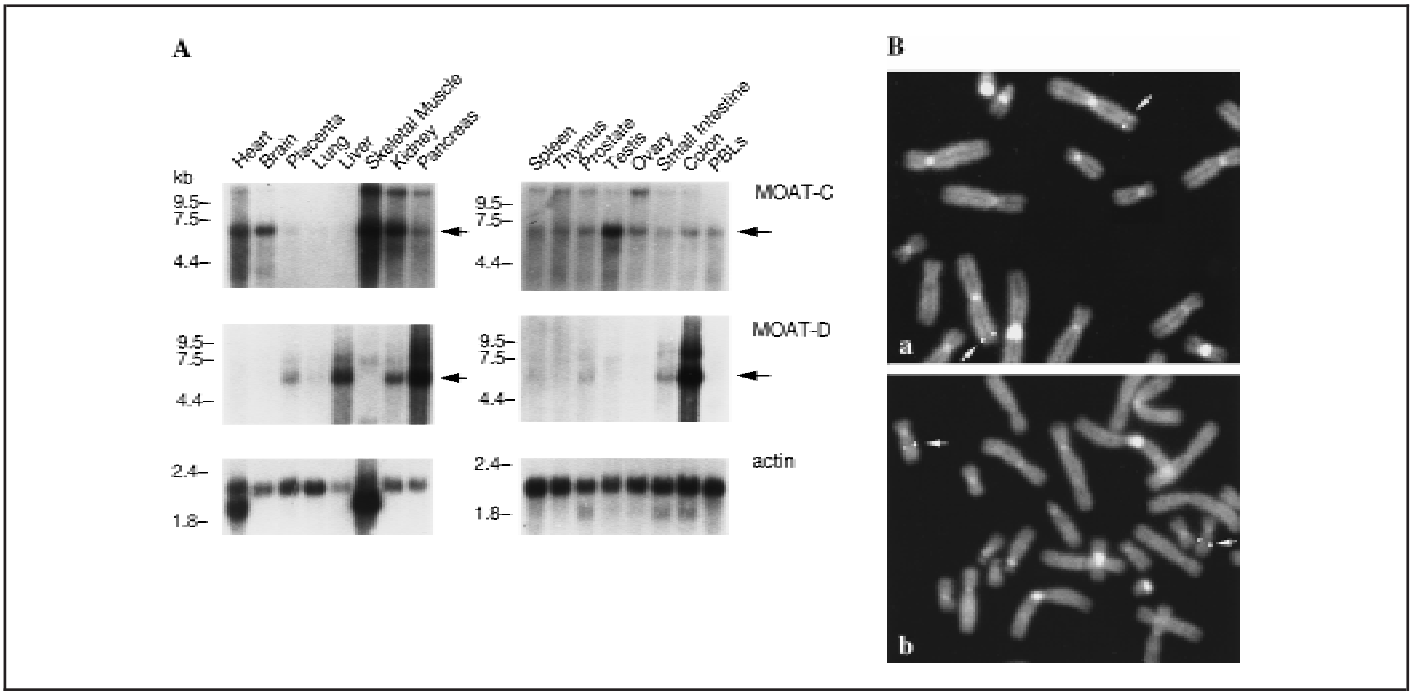

Fig. 3. RNA Expression patterns and chromosomal localizations of MOAT-C and MOAT-D genes. **A)** Tissue distribution of MOAT-C and MOAT-D gene transcripts. Blots containing poly A^+ RNA prepared from various human tissues were hybridized with complementary DNA (cDNA) probes of MOAT-C, MOAT-D, and actin. Arrows indicate the position of the MOAT-C **(top panel)** and MOAT-D **(middle panel)** transcripts. **Bottom panel** shows the control actin transcript. **B)** Chromosomal localization of MOAT-C and MOAT-D genes. Biotin-labeled MOAT-C **(panel a)** and MOAT-D **(panel b)** cDNA probes were hybridized to human lymphocyte metaphase spreads and detected by fluorescein isothiocyanate-conjugated avidin. Hybridization signals at chromosome 3q27 in two metaphase spreads **(panel a)** and chromosome 17q21.3 in two metaphase spreads **(panel b)** are indicated by arrows.

YCF1, a closely related transporter, and SUR, a more distantly related protein involved in the regulation of potassium channels *(28)*. The second group of MRP/cMOAT-related transporters is composed of MOAT-B and MOAT-C. Like MOAT-D, MOAT-B and MOAT-C are more closely related to MRP (39.4% and 35.8%, respectively) and cMOAT (36.8% and 36.2%, respectively) than to other human transporters. However, they share considerably less similarity with MRP, cMOAT, and MOAT-D than the latter three transporters share with each other. In addition, MOAT-B and MOAT-C do not have N-terminal membrane-spanning domains, and their topologies are therefore more similar to those of many other eukaryotic ABC transporters that also have only two membrane-spanning domains (e.g., MDR1 and CFTR).

The functions of MOAT-B, MOAT-C, and MOAT-D remain to be elucidated. Both MRP and cMOAT transport glutathione, glucuronide, and sulfate conjugates. Conservation of the MRP/cMOAT substrate specificity is more likely for MOAT-D than for MOAT-B or MOAT-C, given the higher degree of amino acid identity of MOAT-D. One possibility is that MOAT-D may transport a class of organic anions that is distinct from the conjugates transported by MRP and cMOAT. For MOAT-B and MOAT-C, it is difficult to speculate at this time on the implications of the absence of the N-terminal membrane-spanning domain, since the contribution of this domain to substrate specificity and function has yet to be established in MRP and cMOAT. However, the observation that this structural feature is found in all of the known organic anion transporters suggests that it may be a structural hallmark of organic anion transporters. The only known exception to this is SUR. Thus, the absence of an N-terminal membrane-spanning domain in MOAT-B and MOAT-C, as well as their weaker amino acid similarities to MRP and cMOAT, suggest that their functions and substrate specificities may be different from the latter two transporters. The expression patterns of the three MRP/cMOAT homologues also suggest that their physiologic functions are distinct from those of MRP and cMOAT. We previously found that the MOAT-B transcript is highly expressed in prostate but is also expressed in many other tissues *(15)*. In this study, we found that MOAT-D is predominantly expressed in the colon, pancreas, liver, and kidney. This pattern is distinct from that of MRP, which is widely expressed *(29)*, and cMOAT, which is highly expressed in the liver but is also expressed in the kidney and small intestine *(10,11,30)*. In contrast, MOAT-C is expressed at highest levels in skeletal muscle, kidney, testis, heart, and brain and is also expressed in most other tissues but is barely detectable in the lung and liver. Further studies designed to define the substrate specificities of these MRP/cMOAT-related transporters should help to elucidate their physiologic functions as well as their possible contributions to cellular resistance to cytotoxic drugs and the pharmacokinetics of these agents.

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NOTES

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